BIOLOGICAL PRODUCTS

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BY

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PREFACE

THE author has lectured on Biological Products in his discourses before students during the last almost quarter of a century. He has written upon the subject in different periodicals. Requests have been numerous by graduates of pharmacy, chemistry, medicine and the allied professions for a small volume dealing with these products. It is to answer this demand that this book is presented. Pharmacists, chemists, physicians and others who of necessity are concerned with a consideration of Biological Products may find that a correct understanding of their manufacture, standardization and use will be helpful to them.

Many advances have been made in the production of Biological Products. New methods have been introduced in the manufacture and standardization of the older products and new preparations have been presented. There has been no attempt made in this volume to consider the different theories of immunity. A consideration of the Biological Products only is presented, so as to make available in one volume a connected and concise account of our present knowledge of the more important Biological Products in use. Few details concerning immunity may be found, but they are brief, yet adequate for a better understanding of the subject at hand.

Exhaustive treatment of all Biological Products would require more pages than allotted here. The necessity for limitation in a first edition imposes a restraint which is easier to disregard than to comply with. The scope of the subject matter therefore has been limited to include details where such appear desirable and generalities only are given when considering products used but infrequently. Unimportant details and references other than those which have appeared important have been omitted. Of necessity illustrations have been kept down to a minimum.

The author herewith expresses his deep gratitude to those who have lightened his labors by innumerable kindnesses. In particular, the author wishes to express his appreciation to the following pharmaceutical manufacturing firms: Sharp & Dohme, Philadelphia, Pa.; Eli Lilly and Company, Indianapolis, Ind.; Parke, Davis & Company, Detroit, Mich.; National Drug Company, Philadelphia, Pa.; Wm. S. Merrell Company, Cincinnati, Ohio; and Cutter Laboratories, Berkeley, California, for their kindness in supplying the illustrations of biological manufacture which have been reproduced in this volume.

L. G.

Dedicated to

RANDLE C. ROSENBERGER, M.D.

Scientist-Teacher-Humanitarian-Friend

CONTENTS

BIOLOGICAL PRODUCTS

I

BIOLOGICAL PRODUCTS

Preparations Employed in Serum and Vaccine Therapy

THE preparations considered here being of a complex biological nautre are frequently spoken of as *Biological Products,* a designation which serves as a convenient classification to differentiate such products from the mineral and vegetable groups in our materia medica. Their therapeutic use depends on the numerous phases of immunity and it is of import to understand this intimate relationship between the two.

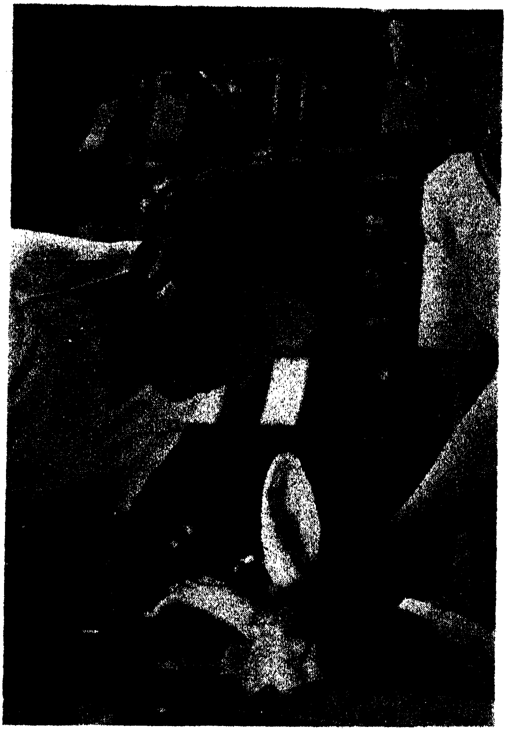
GENERAL CONSIDERATIONS

● Biologic therapy as now practised embraces the prophylaxis and treatment of disease by agents marketed under any of the following titles: *(1) Bacterial Vaccines* (known also as Bacterial Antigens, Bacterins or Opsonogens); *(2) Sensitized Bacterial Vaccines* (so-called Serobacterins); *(3) Antimicrobic Sera* (Antibacterial, Therapeutic or Immune Serums, or Antiserums); *(4) Modified or Attenuated Viruses* (as so-called Smallpox Vaccine and Rabies Vaccine); *(5) Antitoxins* (Antitoxic Serums); *(6) T-A Mixture; (7) Immunizing Toxins; (8) Toxoids or Modified Toxins; (9) Tuberculins; (10) Phylacogens* and *Bacterial Culture Filtrates; (11) Aggressins* (natural tissue fluids from artificially infected animals); *(12) Immunogens* (or Ectoantigens); *(13) Antivenin; (14) Bacteriophage; (15) Extracts or Allergens* (bacterial, animal or vegetable or with specific designations as Pollen Extracts or Leucocyte Extract, etc.); *(16) Normal Serum;* and *(17) Diagnostic* and *Biological Reagents.*

DEFINITIONS

● The following definitions as given in Rule 7 of the *Regulations for the Sale of Viruses, Serums, Toxins and Analogous Products in the District of Columbia and in Interstate Traffic* (Misc. Pub. No. 10, U. S. Public Health Service, Feb. 25, 1935) are of interest: "For the purpose of these regulations, viruses, serums, toxins, antitoxins, and analogous products applicable to the prevention or cure of diseases of man are referred to as biologic products and defined as follows: *I.* A *virus* is a product containing the minute living cause of an infectious disease. *II.* A *serum* is the product obtained from the blood of an animal by removing the clot or clot components and the blood cells. *III.* A *toxin* is a product containing a soluble substance poisonous to laboratory animals or to man in doses of 1 milliliter or less of the product, and having the property, following the injection of non-fatal doses into an

animal, of causing to be produced therein another soluble substance which specifically neutralizes the poisonous substance and which is demonstrable in the serum of the animal thus immunized. *IV.* An *antitoxin* is a product containing the soluble substance in the serum or other body fluid of an immunized animal which specifically neutralizes the toxin against which the animal is immune. *V.* A product is analogous (a) to a *virus* if prepared from a virus, including micro-organisms actually or potentially virulent; (b) to a *serum,* if prepared from some protein constituent of the blood and intended for parenteral administration; (c) to a *toxin* or *antitoxin,* if intended, by parenteral administration, for the prevention or treatment of disease through specific immunization."



Injecting rabbit intravenously for the production of therapeutic sera.

FEDERAL CONTROL

● Biological Products are generally given by parenteral administration. They must, therefore, be free from contamination. Many of them are employed at a time when an active product is desired. It is, therefore, necessary to guard closely the potency of such preparations, providing a potency test is applicable. Vaccines, serums, and analogous products have been deemed of such importance by the Federal government, that their production for interstate sale within the jurisdiction of the United States has been under the special protection of an act of Congress, approved July 1, 1902, antedating even the Pure Food and Drugs Act. Under this law, all establishments producing such products for interstate sale must be licensed by the Secretary of the Treasury upon the recommendation of the Surgeon General of the U. S. Public Health Service. The production is controlled by regulations drawn up by a board composed of the Surgeons-General of the United States Public Health Service, the Army, and the Navy. These regulations provide for repeated inspections of the producing laboratories, for proper labelling and for all other safeguards, which may be required. In practice, the National Institute of Health guards these products. Licenses are valid until suspended or revoked by the Secretary of the Treasury. A list of the establishments holding licenses issued by the U. S. Treasury Department is to be found in Reprint No. 1734 from the Public Health Reports.

Biologicals for veterinary use prepared for interstate sale in the United States must receive a license issued by the Bureau of Animal Industry.

LICENSING

● The U. S. Public Health Service (National Institute of Health), to whom application must be made, makes inspections and examinations of all facilities and methods of procedure in manufacture, and after having found all samples obtained in the plant and on the open market of a high standard of purity, recommendations for a license are made. These licenses are then issued at a nominal charge for periods of one year and are revokable at any time. Licenses for new products are not granted without satisfactory evidence of therapeutic or prophylactic efficiency. Biologicals made and sold or used solely within the states of their origin (no interstate sale) do not come, of course, under Federal supervision, but in the main they are under the supervision of the respective state health departments.

Biologic products imported from foreign countries are refused entry by collectors of customs unless produced in an establishment holding an unsuspended or unrevoked license, or intended for examination prior to obtaining a license. Each foreign establishment holding license and exporting biologic products to the United States is required to file the name and address of one or more representatives in the United States authorized by the establishment to distribute their products, and these representatives must keep records of such distribution. The importation of smallpox vaccine from any foreign country into the United States is prohibited, except smallpox vaccine sent to the National Institute of Health of the United States Public Health Service, where tests are made to demonstrate the absence of any other pathogenic virus.

In Great Britain and in many parts of the British Empire, serums and vaccines are prepared in accordance with the regulations made under the Therapeutic Substances Act of 1931. The Ministry of Health with the cooperation of the Medical Research Council serves in Great Britain in a capacity similar to the National Institute of Health in the United States.

LABELLING AND DATING

● The label must contain the name, address and license number of the manufacturer. An expiration date, laboratory lot number given at the particular time when the product was prepared, and the minimum potency or strength, if such can be determined, are also to be included. The proper name of each product specified in the license must appear on the label and given precedence in position over any trade or other descriptive name adopted by the manufacturer. The Federal law requires that each package of any of the biological products should be stamped with an *expiration date* ("the date beyond which the contents cannot be expected beyond reasonable doubt to yield their specific results"), or date of manufacture or issue. The date of manufacture for products for which: (1) an official standard of potency exists is the last date of satisfactory passing a potency test; (2) no official standard of potency exists, the date of removal from the animal in case of animal products or the date of cessation of growth in the case of other products; or (3) in the case of products used for specific desensitization, the date of extraction is designated. The date of issue from cold storage is to be accepted in lieu of the date of manufacture provided such date is not more than 2 years after date of manufacture if the product is kept constantly below 0°C., not more than 1 year if kept constantly below 5°C., or not more than 3 months after date of manufacture if the product is kept constantly below 15°C.

STORAGE

● Many of the biological products deteriorate very rapidly if kept at room or higher temperatures. This is especially true of modified smallpox virus, modified rabies virus (so-called smallpox and rabies vaccines) and other preparations, as the antitoxins and antimeningococcic and antipneumococcic sera. The method of determining the *exact* and *accurate* expiration date is really not under the control of the manufacturer or the government, as the temperature at which these products are stored after leaving the manufacturer's control cannot be regulated.

The temperature at which biological products are stored, particularly rapidly deteriorating preparations is very important. Pharmacists and others should, therefore, keep their biological products in cold storage, at as low a temperature as possible, (4°C.), being sure that the latter never exceeds 15°C. Freezing, however, should be avoided, except in the case of modified smallpox and rabies viruses. The latter should be kept as cold as possible, the colder the better.

POTENCY TESTS

● Official potency standards are made or official potency tests have been established by and are conducted at the National Institute of Health for:

Antitoxins—all forms of Diphtheria Antitoxin, Tetanus Antitoxin, Botulinus Antitoxin type A, Botulinus Antitoxin type B, Perfringens and Gas Gangrene Antitoxins, Vibrion Septique Antitoxins, Staphylococcus and Scarlet Fever Streptococcus Antitoxins.

Serums—Antidysenteric Serum (Shiga), Antimeningococcic Serum and Antipneumococcic Serum (Types I and II). Types V, VII and VIII are being controlled.

Bacterial Vaccines—Typhoid and Triple Vaccines prepared from the typhoid or typhoid, paratyphoid A and paratyphoid B bacilli.

Toxins or Modified Toxins—Diphtheria Toxin-Antitoxin Mixture, Diphtheria Toxin for the Schick Test, Diphtheria Toxoid, Scarlet Fever Streptococcus Toxin for the Dick Test and for Immunization, and Tetanus Toxoid.

Standard samples for comparison of products other than those mentioned above may be distributed by the National Institute of Health. Manufacturers are requested to forward samples from each lot of such marketable preparations and to withhold distribution pending receipt of release. The latter is made on the basis of protocols furnished by the manufacturer and of tests made. This is to insure the safety and potency of products for which official methods of testing have not been made available.

Tests for potency are made after the completion of all the processes of manufacture except filling the final containers. In case of products for which an official standard of potency has been adopted, the potency must be expressed on the label in terms of the official standard. Where an official standard of potency has not been adopted and an official test is not made prior to the release of the preparation for sale, the label must bear the statement: "No U. S. standard of potency." The latter provision does not apply to products specifically indicated under their respective headings, as Modified Smallpox and Rabies Viruses. A Permanent Commission of the Health Organization of the League of Nations is attempting to coordinate and improve existing methods of standardization so that there may be more uniformity.

In accordance with the provisions of section 63 of the "*Regulations for the sale of viruses, serums, toxins and analogous products, approved*

January 1, 1937," the datings in the United States for all licensed biologic products are as given below.

Any lot of any product shall be subject to recall, regardless of dating, upon the discovery of lack of purity or potency, or if it is found to be subject to undue deterioration.

EXPIRATION DATE

● For the purpose of fixing the *expiration date* (the date beyond which the contents of the package cannot be expected beyond reasonable doubt to yield their specific results), the date of manufacture shall be as follows:

1.—For products for which an official standard of potency exists, or which are subject to official potency tests, the latest date of passing satisfactory potency tests.

2.—For products for which no official standard of potency exists and which are not subject to official potency tests, the date of removal from the animal in the case of animal products, or the date of cessation of growth in the case of other products, except products used for specific desensitization, in which case the date of extraction shall be used.

The *date of issue* shall, unless otherwise specifically provided, be not more than 3 months after date of manufacture if the product is kept constantly at a temperature not exceeding 15°C., or not more than 6 months after the date of manufacture if the product is kept constantly at a temperature not exceeding 10°C., or not more than 1 year after the date of manufacture if the product is kept constantly at a temperature not exceeding 5° C., or not more than 2 years after date of manufacture if the product is kept constantly at a temperature not exceeding 0°C.

The dates beyond which the different products cannot be expected, beyond reasonable doubt, to yield their specific results (expiration dates) shall be not later than the following:

Antitoxins (with official standards, or subject to official potency tests)—One year after date of manufacture or date of issue, with a 20 percent excess of potency; 2 years with a 30 percent excess; 3 years with a 40 percent excess; or 4 years with a 50 percent excess.

Antitoxins (without official standards and not subject to official potency tests)—One year after date of manufacture or date of issue.

Antidysenteric Serum—Eighteen months after date of manufacture or date of issue.

Antimeningococcic Serum—Six months after date of manufacture or date of issue.

All Other Immune Serums—One year after date of manufacture or date of issue.

Non-immune Serums—Three years after date of manufacture or date of issue.

Bacterial Vaccines, Sensitized Bacterial Vaccines, Venoms and Modified Bacterial Products—Eighteen months after date of manufacture or date of issue.

Toxoids—Two years after date of manufacture or date of issue.

For Smallpox Vaccine, Rabies Vaccine, Tuberculins, T-A Mixture, Scarlet Fever Streptococcus Toxin, and Diphtheria Toxin—See under respective headings.

Leucocytic extract—one year after date of manufacture or date of issue.

Pollen Extracts, Animal Epidermal Extracts, Animal Food Extracts and Vegetable Food Extracts—For extracts containing no glycerin, 18 months after date of manufacture or date of issue. For extracts containing at least

50 percent glycerin, the date of issue shall be not more than 4 years after the date of manufacture if the product is kept constantly at a temperature not exceeding 10°C.

Animal Oil Extracts and Vegetable Oil Extracts—Five years after date of manufacture or date of issue.

Fungus Extracts—Three years after date of manufacture or date of issue.

Countries other than the United States impose dating regulations. In many instances the U. S. regulations covering dating are the same as in such foreign lands. In some instances more liberal regulations are imposed in foreign countries and in a few instances there are no regulations covering biological products.

ADDED PRESERVATIVE

● The bacterial vaccines are prepared under aseptic conditions and sterilized by heat. The other products are prepared under aseptic conditions and forced through a Berkefeld or similar type filter so as to be assured of sterility. With all these strict precautions, it has been found advisable to add an antiseptic, so as to have an additional safeguard as a preventive against contamination in the last stages of handling. The small amount of antiseptic present possesses no deleterious effect. The most commonly used antiseptic is cresol (0.25 to 0.4 percent). Phenol (0.5 percent), glycerin, organic mercury compounds (merthiolate and phenyl mercuric acetate) and chloretone are also used. None is ideal for all purposes. The mercurials appear to be unsuited in bacterial vaccines and as preservatives for serum to be concentrated and refined. The efficiency of a preservative varies with the character of the product. Those in use at present do not affect the potency of biological products. In a recent study it was found that considerable amounts of phenol were carried down or adsorbed by protein in the preparation of certain products. Caution must be exercised in those instances in the addition of phenol to the finished product (*J. Lab. & Clin. Med., 24,* 137 (1938)).

In preparations which are employed in large volumes, the composition and amount of the preservative are obviously of some importance. Liquid serum must not contain more than 20 percent total solids, nor more than 0.5 percent of preservative. Products intended to be used intraspinally or intravenously must be clear, free from excessive coloration, or excessive viscosity, and those to be used intraspinally must contain not more than 0.35 percent of preservative. Containers of products intended to be used intraspinally or intravenously must be of such material that the presence of objectionable color or of sediment in the contents may be readily detected.

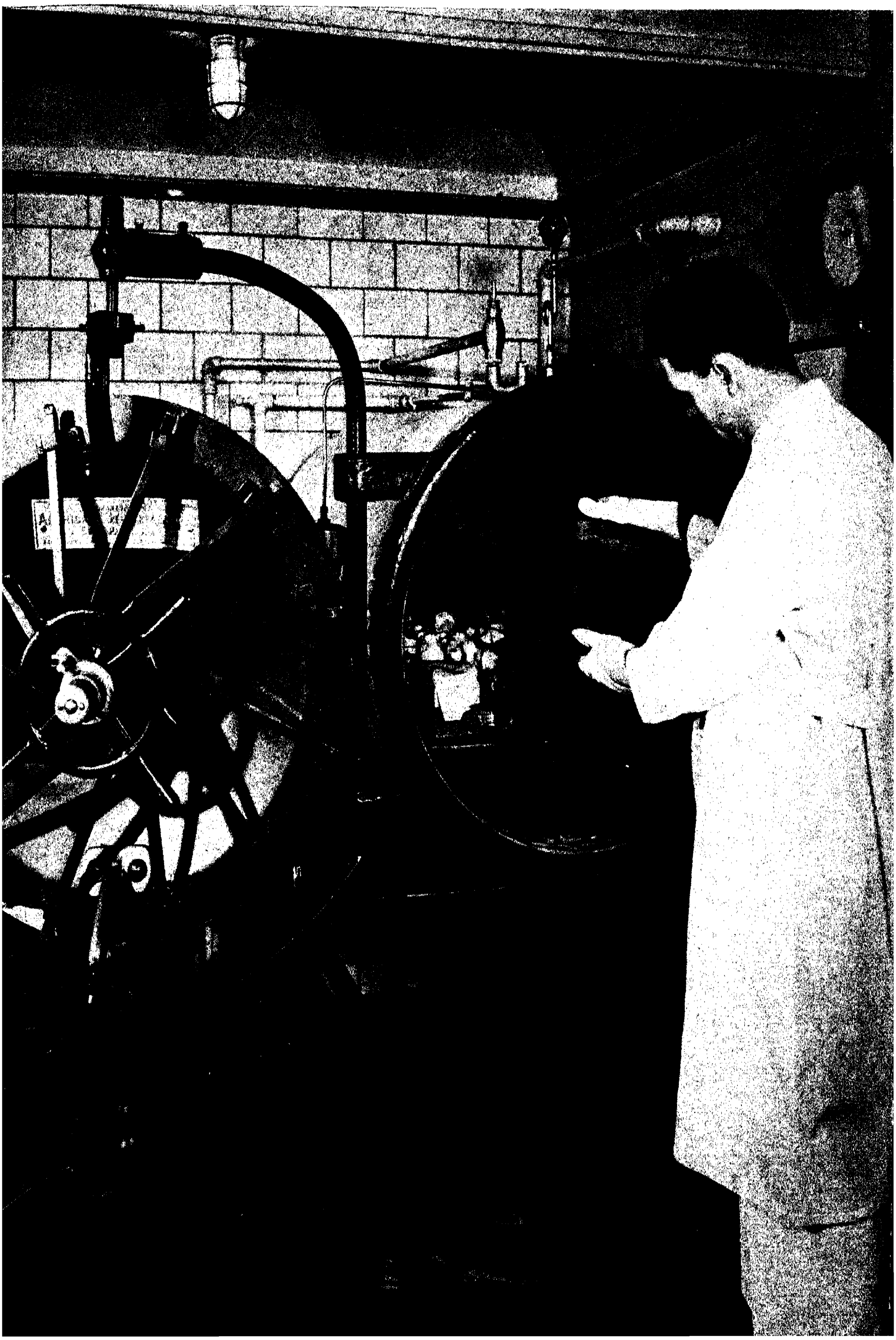
TESTS FOR PURITY AND SAFETY

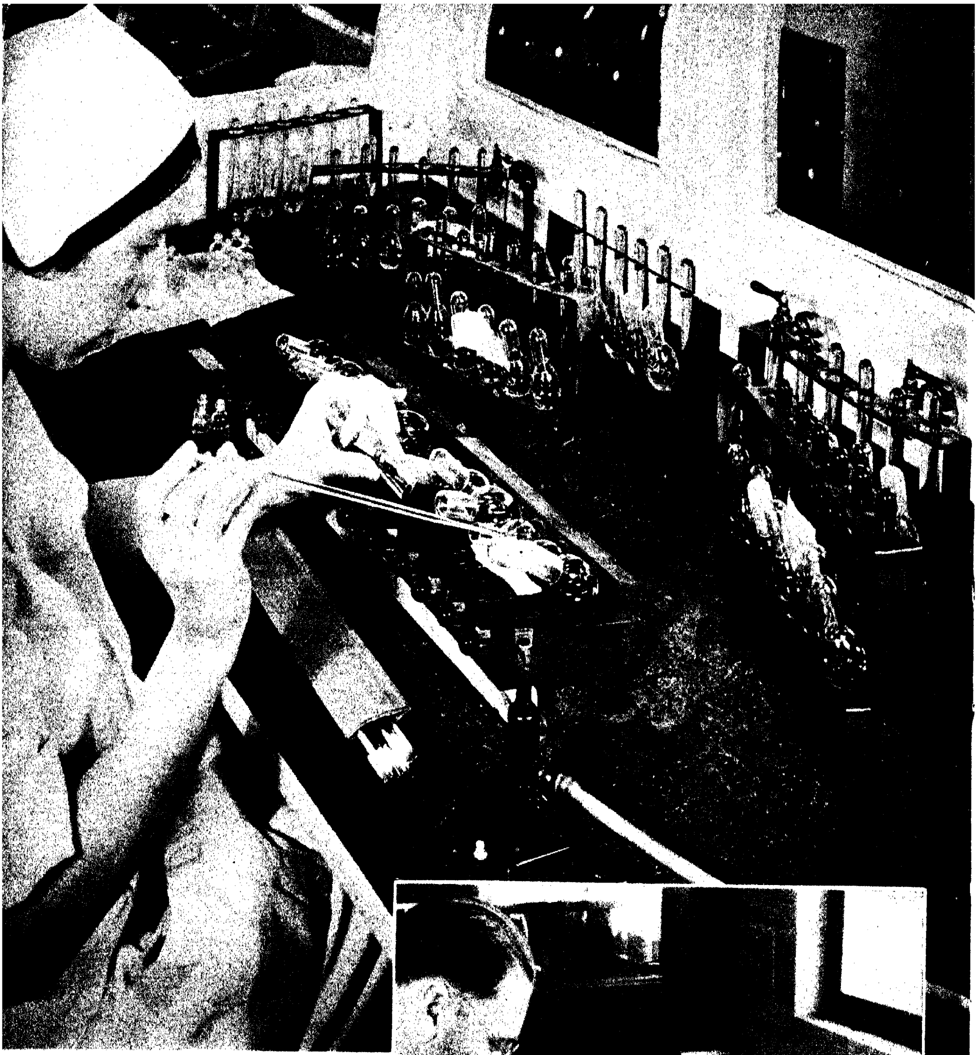
● All biological products, though they have been sterilized, as previously mentioned, must be tested for their purity before they are marketed. This generally consists of:

1. A pathogenicity test—by injecting a portion of the material into guinea-pigs and if necessary into other laboratory animals. The latter are to be kept under observation for two weeks, to detect the presence of any foreign toxic substance.

2. A cultural test—culturing in suitable media for the accidental presence of bacteria is to be made at each stage and at the end of the preparation of the various products. This includes an aerobic and an anaerobic culture (fermentation test). More detailed considerations are given in the following pages.

Sterilizing biological containers.







Testing sterility of finished biological products.

TESTS FOR IDENTITY

● Samples of each lot of all products are not only tested for safety but some reasonable test of identity is made after the labels have been affixed to the final container, providing such test is applicable. For instance, serological tests are possible for diphtheria antitoxin or typhoid vaccine.

OPENED PACKAGES

● On account of the numerous opportunities for contamination and the possibility of subsequent infection, no portion of any of the biological products in a container, especially those from which the stoppers have been removed, should be kept for future use, after the container has been opened. It is advisable to use the whole contents *AT ONE TIME* in one or more cases, unless suitable rubber-capped containers are employed when the needle can be plunged through the previously disinfected rubber stopper, or unless great care and asepsis is observed at each occasion when a portion is to be removed.

PERMANENT RECORDS AND OTHER REGULATIONS

● Permanent records must be kept of each batch of material showing every step. Laboratories must be kept under the best sanitary conditions at all times. All animals used must be properly housed and well cared for. Complete necropsy records must be kept of each animal that dies or is killed.

Spore bearing pathogens are not to be kept in the same laboratory with the other cultures. All bacterial cultures are examined frequently for purity and efficacy. The admission of visitors must be limited as far as possible.

It is understood that the National Institute of Health does not endorse in any way the therapeutic value of products manufactured by those who are granted licenses. It merely attempts to control their manufacture and marketing so that they are free from extraneous contamination and sees that the public is getting that for which it is paying.

PRESERVING AND MARKETING BIOLOGICAL PRODUCTS IN THE DRIED STATE

MANY significant contributions have been made in an attempt to dry antisera and other biological products as the superior keeping qualities of such dried products were recognized by many workers. A few biological products are available in the dried state as marketable preparations. Simple desiccation however has many limitations. Only small quantities can be dried by existing dehydrating apparatus. The end product may be found to be imperfectly or only slightly soluble in water. There may even be a partial denaturation of the product during the drying process. A detailed account of the desiccation of sera and other biological products in the frozen state with the preservation of the original qualities of the products so treated is given by Elser and Collaborators (*J. Immunol.*, *28*, 433 (1935)).

LYOPHILE PROCESS

● Flosdorf and Mudd (*J. Immunol.*, *29*, 389 (1935)) described a procedure and apparatus for the preservation of biological products in desiccated form. The method is essentially one of rapid freezing at a very low temperature (below freezing point) and rapid dehydrating from the frozen state under high vacuum. The operation is continuous and is conducted in the final

containers in which the material is to be sealed under the original vacuum, stored and distributed. The resultant product is a porous solid occupying practically the same space (volume) as the liquid (serum) from which it was prepared. It is claimed that the potency of the product suffers no detectable loss in processing and the rate of subsequent deterioration is reduced to a small fraction of that in the liquid state. On the addition of distilled water, the porous product redissolves readily. The term "*Lyophile*" (solvent-loving) referring to its remarkable and complete solubility has been applied to these products by one of the large biological laboratories using this process.

Apparatus to prepare products in "lyophile" form is available for use in research laboratories, for board of health and hospital work and for industrial scale production. Normal and convalescent human sera, animal antisera, enzymes, viruses, toxins, miscellaneous proteins and other materials have been preserved unaltered by this procedure for long periods of time. This process is employed at present commercially for the storage of antisera, antitoxins and other products in bulk and indications are that lyophile processing will be employed extensively in the near future in the production, manufacture, and even marketing of many biological products. Bacteria treated by lyophile processing appear to remain viable when stored at room temperature. They retain their virulency for indefinite periods of time.

One of the large biological manufacturing laboratories recently placed on the market a complete line of lyophilized antisera, under the trade-mark name "LYOVAC." In the marketed package the lyophilized serum is found in one container and in another sterile vial, sterile distilled water is present, to be used to bring the dry product into solution.

CRYOCHEM PROCESS

● Flosdorf (*J. Immunol.*, *34*, 469 (1938)) described recently a procedure (cryochem-process) and apparatus for the preservation of biologicals by desiccation in vacuo from the frozen state and claimed to be at a lower cost of operation than heretofore. Water vapor is removed by a specially prepared calcium sulfate which may be regenerated by heat at atmospheric pressure. This process and apparatus may be used for all the purposes for which the lyophile process is of value.

SANITARY STANDARDS FOR MANUFACTURING ESTABLISHMENTS

OFFICERS of the United States Public Health Service usually appear unannounced and inspect licensed establishments. They investigate thoroughly everything connected directly or indirectly with the manufacturing process. Frequently they communicate details of sanitary standards, methods of manufacturing and testing, and methods of keeping records and other information. The following are among some of the regulations thus communicated:

REGULATIONS OF BIOLOGIC PRODUCTS

(Memorandum of Details Under Section 36, Nat'l. Inst. Health)

● The admission of visitors should be limited as far as possible, and should at no time be such as to endanger the thoroughness of any process. Precautions should be taken to lessen dust both inside and outside the laboratory, by oiling the roads and by frequent moist cleaning of floors and walls. Particular care should be exercised to avoid the tracking of outside dirt into rooms where sterile operations or the preparation and handling of vaccine virus take place. The changing of shoes or the wearing of cloth foot-casings is advised.

In establishments of considerable size, a competent bacteriologist without other routine work, should be charged with the supervision of sterility, and should be obliged to give attention from time to time to different parts of the procedure, to the end that both personnel and methods be as efficient in this respect as possible.

Sterilization of Equipment

● Ordinarily two hours of dry heat sterilization at 170° C., or thirty minutes of steam sterilization at 15 lbs. pressure, are considered adequate to kill spores unless the material is particularly impermeable or dangerous. In all sterilization it is important that the goods be loosely arranged, with free vertical air channels. In dry heat sterilization the lower part of the oven is frequently found to give inadequate heat as compared with the remainder. Autoclaves should be exhausted by a thorough preliminary vacuum before the steam is admitted and the degree and duration of temperature recorded by automatic devices which are checked frequently to insure that they are recording properly. To insure sterilization and to maintain thorough asepsis in assembling and use, the various pieces of apparatus should be of as simple construction as practicable.

Containers and apparatus should be sterilized as fully assembled as possible in order to minimize handling after sterilization; yet care must be taken to secure thorough permeation by the hot air or steam. In some cases rubber goods may be sterilized by boiling for half an hour in three to five per cent phenol, or similar disinfectant.

Care of Horses

● There should be a sufficient number of hostelers to keep the horses well groomed and comfortably cared for. The horses should be marked plainly or numbered so as to be identified readily. All horses should be disposed of which are seriously and permanently disabled, especially those having any difficulty in standing or rising, or those injured so as to interfere with proper care. The stables should be fully lighted and facilities provided so that each horse receives daily inspection sufficient to disclose abnormalities and reactions.

Sterility tests of each batch of toxin or other antigen should be made. All injections should be made with due regard for asepsis, and no subcutaneous injections made within 6 inches of the bleeding site.

No horse should be bled with persistent general reaction or with local reaction near the point of bleeding. The use of the flame technic is advised in the handling of blood, plasma, and serum.

Filling Containers

● As far as is practicable, bottles for bulk storage of an unfilled product should be of such size that one trip to the filling room will suffice.

Filling biological products into final containers should be performed so as to minimize the dangers of finger and dust contamination. Containers having an opening at one end should be sterilized by either dry heat or steam, arranged in racks with the opening pointing down, and in cases, so that they will be protected from dust when removed from the sterilizer and yet be permeable to the hot air or steam. Such an orderly arrangement of the containers and a similar arrangement of caps or stoppers conduces to uniform asepsis in filling, since the part of the container or stopper likely to come in contact with the product is pointed down and not handled in filling. Only as many containers, or parts, as are likely to be used at one filling should be sterilized in a single package. In any case a previously opened package should never be used without resterilization.

In setting up filling apparatus and generally in sterile procedures the use of the Bunsen flame is advised. The room for filtering should be protected from dust and not used for other purposes. The room should be comfortable and with sufficient air supply to render unnecessary the opening of windows in warm weather. Artificial cooling is advised to this end. It is well occasionally to ascertain the dustiness of the room by exposing plates on the filling table and also over the air inlets. A glass shield with open front under which the filling can take place, with opening for the burette or other filling apparatus, and the use of sterilized gloves are also advised.

Tests for Identity

● Before making each batch of bacterial vaccine or similar product, the culture should be plated or streaked, and isolated typical colonies selected. Tests sufficient to establish the identity of the pure strain should then be made, or at least every three months if lots are prepared more often. The trimonthly tests should each comprise salient points sufficient to establish the species in general, and be varied so that a practically complete identification of the strain will have been carried through in the course of the year. If the scientific personnel of the establishment is not sufficient for this purity identification, the number of different bacterial vaccines manufactured should be reduced.

Tests to establish identity should be made on each lot of antisera, and the antigens used in the production of these sera should be controlled as stated in the preceding paragraph for bacterial vaccines.

Tests for Harmlessness

● Bacterial tests are required to show sterility as regards organisms which will grow at ordinary incubator temperatures, for all products except vaccine virus. Preliminary tests on various media such as glycerin agar, ascitic or blood agar, etc., should be made when the organism used in the

manufacture of the product will not grow readily on ordinary media.

The medium which is in use at the National Institute of Health for testing the sterility of all products and which is recommended for routine testing is made as follows:

To eight kilograms of ground fresh meat freed from fat, sixteen liters of distilled water are added and the mixture infused in the ice chest twenty-four hours. Sixteen liters of juice are squeezed out through cheese cloth, heated in streaming steam for one hour, autoclaved at fifteen pounds pressure for thirty minutes, filtered through moistened paper and brought up to the original volume. Five grams of sodium chloride per liter and ten grams of peptone per liter are added and the broth stirred until solution takes place. The pH is adjusted to such a point as experience shows will result in a pH of 7.5 in the final broth in the fermentation tubes by adding solution of sodium hydroxide. The broth is heated in streaming steam for thirty minutes, filtered through moistened paper, placed in glass-capped Smith fermentation tubes, each containing at least 25 cc. and holding a seal of at least one centimeter in the open arm, and autoclaved at fifteen pounds pressure for twenty minutes. The Smith fermentation tubes should be in racks which facilitate tipping oxygen bubbles out of the long arm when hot, or which allow such bubbles to flow out of the tubes while the heating is going on, and which permit ready inspection of all parts of the tube for growth. The design in use at the National Institute of Health is recommended. If the broth is not to be used immediately, it may be filtered into flasks containing not more than 1,500 cc. each, sterilized by streaming steam for two hours or by autoclaving for twenty minutes at fifteen pounds pressure, and stored prior to filling into fermentation tubes.

To detect contamination, the fermentation tubes may be incubated for a few days, or an adequate number (about 20%) of controls planted with material known to be sterile, simultaneously with the test. Not more than five hours before planting, the fermentation tubes shall be heated to fully 100° C. for thirty minutes and immediately tipped to expel the air from the long arm unless the tubes are arranged in the sterilizer so that the bubbles leave each tube while being heated. The pH at this point should be between 7.2 and 7.8, but instead of repeated adjustments of reaction during the process of preparation of the medium, it is preferable to add enough alkali in the beginning to insure a proper reaction when all the steps are completed. The amount to be added can be ascertained only by repeated trials, using the same ingredients. No acid should be added at any point in the process.

No dextrose need be added to the batch of medium provided a preliminary test has shown that it contains an appreciable amount of muscle sugar. This test is made by inoculating two Smith fermentation tubes filled with the fully prepared and sterilized medium, with an active strain of colon bacillus. If after over-night incubation both fermentation tubes show a bubble of gas filling the tip of the closed arm, sufficient sugar may be assumed to be present. If not, or in the absence of such a test, approximately 0.03% of dextrose should be added just before the final heating in the fermentation tubes. For this purpose 1% dextrose solution in flasks containing not more than 50 cc. each should be sterilized in the autoclave at fifteen pounds pressure for fifteen minutes, and added to the broth in the proportion of 1 cc. to each fermentation tube.

Sterility Tests, General

● Planting is done within five hours after the broth has cooled. In general, 0.25 cc. of the fluid to be examined is planted in one tube and 1.0 cc. in another tube. The tubes are incubated at 37° C. for seven days and should be examined on the 2nd, 4th, and 7th days after planting, and agitated only after 48 hours incubation to insure initial anaerobiosis. The lowest part of the bend, as well as both arms, should be examined for slight growths.

In case the material is turbid (antirabic virus, etc.) transplants to fresh fermentation tubes, as well as smears, should be made on the seventh day. When testing materials not miscible with the broth culture medium it is suggested that some procedure be adopted to secure solution or emulsification of the material being tested, as for example, the use of 0.5 per cent saponin in broth in testing oils or oily preparations.

Sterility Tests on Bulk Product, Before Filling

● At least 10 cc. shall be planted, prior to bottling, from each bulk container holding more than 1 liter and from bulk containers of less than a liter capacity, at least 3 cc., into enough fermentation tubes so that the coefficient of the preservative shall not exceed that of phenol in a dilution of 1 to 10,000. If the bulk containers are opened at any subsequent manipulation, two 0.25 cc. and two 1.0 cc. fermentation tubes should be planted from each container. If contaminations appear in any of the bulk tests, the test may be repeated, but no lot shall be passed until such test shows no growth, and if the same contaminating organism appears in more than one test, the material shall be discarded or resterilized.

Sterility Tests on Final Containers

● Of the final containers selected at random from each filled lot, three to ten shall be tested in accordance with the following schedule:

Total Number Containers in the Lot	Number of Containers to be Tested
100 or less	3
101 to 150	4
151 to 200	5
201 to 250	6
251 to 300	7
301 to 350	8
351 to 400	9
Over 400	10

At least one 0.25 cc. and one 1.0 cc. fermentation tube shall be planted from each container. In the case of products to be administered in doses larger than 1 cc., the entire dose up to 5 cc. from each container tested shall be planted in fermentation tubes containing enough broth to dilute the preservative so that its coefficient shall not exceed that of phenol in a dilution of 1 to 10,000, but not more than 5 cc. need be planted from any container. In case contaminations appear in any of the tubes planted, the test may be repeated with the same number of containers, but no lot shall be passed until the final test shows no growth throughout, and a lot shall be discarded if the same organism appears in more than one test.

In the case of products made up in small lots or single treatments, such as rabies vaccine made by the Pasteur method, the preliminary material (e.g. cords when removed from the rabbits) shall be tested in two fermentation tubes. Each week a dose shall be made in duplicate, and

the second lot used for test purposes. In case this shows growth, no further doses are to be sent out from this material until a repetition shows no growth. Emulsions of antirabic virus are not to be held pending the result of the sterility test, but records of the tests are to be kept.

Testing Vaccine Virus

● Tests are made on vaccine virus to show the bacterial count, to exclude the presence of tetanus, and to insure potency.

For the bacterial count, the contents of one vaccination outfit are washed under aseptic precautions into a conical glass containing 1 cc. of saline solution. All clumps are broken up by drawing the suspension in and out of the capillary pipette with a squarely broken off point, pressed against the bottom of the container as in opsonic technic. From this suspension one or more agar plates are made. The drops are delivered into Petri dishes, and the melted agar at 45° C. poured in and mixed thoroughly by tilting. Incubation is at 37° C. for 48 hours. It is advisable to count at least eight final containers from each filling.

In the test for tetanus, samples of the virus from each calf shall be tested separately. Only ground glycerinated pulp shall be tested which has been kept without other preservative for at least seven days at a temperature of 10° C. or higher or until the bacterial count is below 50 per vaccination. If phenol or preservation other than glycerin is used, a sample from each calf, without the additional preservative, shall be kept out for this test. Two cubic centimeters of the virus shall be planted in each of four Smith fermentation tubes, each containing at least 25 cc. of meat infusion broth made as for the sterility test. Just before planting, these tubes shall be heated to fully 100° C. for 30 minutes, and while the level of the broth is rising in the closed arm, tilted to remove the oxygen from the closed arm, unless the tubes have been arranged in the sterilizer so that the bubbles leave each tube while being heated. The planting shall take place as soon as the tubes have cooled to a temperature between 45° and 30° Centigrade. The tubes shall be incubated at 37° C. and inspected daily. Each tube showing gas or growth in the closed arm shall be used for inoculation into animals. Mice or guinea pigs shall be inoculated subcutaneously with 1 cc. of the unfiltered broth 24 to 48 hours after the first appearance of growth in the closed arm, and also nine days after planting. These animals are observed daily for six days and if any die within three days without showing symptoms of tetanus, the test shall be repeated immediately by inoculating 0.1 cc. from the same fermentation tube. The virus from any calf shall be discarded if symptoms of tetanus appear in any mice inoculated therewith.

It is suggested that manufacturers also make use of the following medium, since it has been found equal and probably superior to the usual sterility broth in the detection of tetanus:

Beef muscle is ground in a meat grinder and weighed. Two parts of water are added to one part of the ground beef and the mixture cooked in the Arnold sterilizer for one hour, stirring occasionally. It is then filtered through filter paper and allowed to drain for one hour. Both meat residue and filtrate are saved. The pH of the filtrate is adjusted to 8.5. About five grams of the meat residue are placed in each tube and 8-10 cc. of the broth are added. The tubes are then sterilized at fifteen pounds pressure for one and one-half hours. The following day a cap of sterile petrolatum (petroleum jelly, vaseline) is added to each tube and the tubes heated in the Arnold sterilizer for one and one-half hours.

The medium should be of a rich golden brown color with a final pH of 6.8 to 7.0.

Tubes showing gas or growth should be inoculated into animals in the usual manner.

Each lot should be inoculated cutaneously on rabbits in order to establish potency in comparison with a lot of known high potency on the opposite side of each rabbit, using dilutions of 1:1000, 1:3000, 1:10,000 and 1:30,000 for each lot.

II

ANTIBODIES EMPLOYED AS PROPHYLACTIC OR THERAPEUTIC AGENTS

ANTIBODIES are specific protective substances produced by the tissue cells of a host in reaction against an antigen, as infecting animal parasites, bacteria and their products, or other foreign protein. The term *antigen* (meaning *antibody-producer*) was coined to designate any substance capable of giving rise to the development of specific antibodies in the circulation of animals to which it had been parenterally administered. The number of substances capable of acting as antigens is very large, as practically any foreign protein substance may serve as an antigen. The kinds of specific antibodies produced vary. Some (as *antitoxins*) are directed against or are specific in their power of neutralizing the soluble toxins (as the antigen). The other antibodies present in the different marketed serums are directed against the specific antigen (cellular proteins or endocellular products), as *antibacterial serums* employed against the bacteria themselves. The antibodies in such serums agglutinate or precipitate their specific antigens (and are known as *agglutinins* or *precipitins*), or cause complete dissolution of the specific antigen (and are known as *bacteriolysins*); or they may so alter the antigen as to lower its resistance and render it more easily phagocytable by the body cells (as *opsonins* or *bacteriotropins*). All preparations, the efficiency of which depends upon their antibody content, are employed in passive immunization. The latter (an increased resistance afforded against infection) is designated as passive immunization inasmuch as our own body cells (or the body cells of the one injected) did not produce the antibodies, but our system merely received them passively in the form of an injection of an antibody-laden serum. The antibodies themselves are produced by active immunization in another animal (large domestic animals, usually the horse, though goats and oxen are employed occasionally). These animals are injected with the antigen, the latter causing the production of the specific antibodies. Inasmuch as the blood serum contains most of these antibodies and the serum which is obtained from the blood of the treated animal serves as the usual vehicle by which the antibodies are transferred, this method of treatment is commonly spoken of as *serum therapy*. Serum containing specific antibodies of value for immunization (even only passively) is known as *"Immune Serum"* to differentiate it from *"Normal Serum"* which does not contain antibodies.

From the practical standpoint, Immune Serums are employed to produce two main types of passive immunization: (a) antitoxic immunity, resulting from the injection of antitoxins which antagonize, or are directed against, or neutralize the true or extracellular toxins (as in the use of Antitoxins); and (b) antibacterial immunity, resulting from the injection of agglutinins, precipitins, bacteriolysins and bacteriotropins which are directed against the bacteria themselves (or cellular proteins or endocellular products) as in the use of Antibacterial Serums. The Antiviral Serums (as antipoliomyelitis serum) are in the main comparable to the Antibacterial Serums.

ANTITOXINS

THESE are the most important of the biological products and the most useful of the preparations depending upon specific antibodies for their activity. As the name implies, they neutralize or counteract toxins. Though soluble toxins are secreted by members of the animal kingdom (known as *zootoxins*) as the venoms of snakes, scorpions, etc., and soluble toxins are secreted by plants (so-called *phytotoxins*), it is the extracellular toxic products of bacteria, which are most frequently referred to as the typical soluble toxins. The diseases produced by the latter are serious and the prevention or cure of these conditions depends upon the neutralization of such soluble toxins. Bacteria excreting extracellular toxins and causing dangerous diseases are: *Corynebacterium diphtheriae,* causing diphtheria; *Clostridium tetani,* causing tetanus or lockjaw; *hemolytic streptococci* from cases of scarlet fever (so-called *Streptococcus scarlatinae*); *hemolytic streptococci from* cases of erysipelas; *Clostridium botulinum* causing botulism; *Clostridium welchii* causing internal disturbances and gangrene; and other species of the genus Clostridium, pathogenic anaerobes associated with wounds as will be mentioned under Gas Gangrene Antitoxin.

DEFINITION

● *Antitoxins (briefly defined) are the sera (or generally the globulins from sera dissolved in normal salt solution), obtained from the blood of animals, which have been immunized against specific extracellular toxins, and, therefore, containing antibodies, known as antitoxins, which will neutralize these toxins. The antitoxins, so prepared, are specific, i.e. they will neutralize the particular extracellular toxins which were employed to produce them.*

The methods of preparing the antitoxins do not differ materially in any essential points, with the exception that the toxin from aerobes are produced under aerobic (with access to air) conditions and the toxin from the anaerobes are obtained under anaerobic (without air) conditions. The descriptions of the method of the preparation of diphtheria, tetanus, gas gangrene, botulinus, and scarlet fever streptococcus antitoxins will be given under their respective headings. The other marketable antitoxins are prepared in an almost identical manner with but little modification. Practically all of the marketable antitoxic serums are concentrated and purified. In this procedure, described under Diphtheria Antitoxin, the pseudoglobulin fraction to which the antitoxic substances are attached is obtained in a highly purified state free of most of the other serum constituents. It is this purified antitoxin-carrying pseudoglobulin dissolved in physiological saline solu-

tion which yields the marketable antitoxins. The latter are globulins or more likely associated with the globulin molecules. The strength or potency of antitoxins are recorded in terms of *units*, a unit being a given quantity of antitoxin in any serum or serum concentrate which will neutralize a definite and standard amount of its homologous toxin.

MARKETABLE ANTITOXINS

● The general rule for serum therapy which, of course, includes antitoxins, is that best results are obtained with immune serums if the latter are used *early* in the disease. Injections of normal or immune horse serum or preparations from the latter may produce various reactions and untoward effects frequently spoken of as *"Serum Sickness"* or *"Serum Disease"*. This is regarded as a true anaphylactic phenomenon. It may be necessary to observe certain precautionary measures when administering antitoxic or antibacterial serums. Testing the patient for skin sensitivity and even desensitization may be necessary, as will be discussed under Diphtheria Antitoxin. Diphtheria, tetanus, scarlet fever streptococcus, and gas gangrene (perfringens) antitoxins are the marketable antitoxins most frequently used. The soluble toxins from different organisms are being employed experimentally for the production of antitoxins. The following are other marketable antitoxins which are being advocated: botulinus antitoxin; staphylococcus antitoxin; erysipelas streptococcus antitoxin; puerperal septicemia antitoxin (or antistreptococcic serum); meningococcus antitoxin; and vibrion septique antitoxin, or this combined usually with tetanus and perfringens antitoxins.

OFFICIAL U.S.P. ANTITOXINS

● In the *United States Pharmacopoeia*, there are to be found official the following antitoxins: *Antitoxinum Diphthericum; Antitoxinum Scarlatinœ Streptococcicum;* and *Antitoxinum Tetanicum.* Diphtheria and tetanus antitoxins and *Antitoxinum Welchicum* (gas-gangrene antitoxin) are official in the *British Pharmacopoeia.*

III

ANTITOXINUM DIPHTHERICUM

Diphtheria Antitoxin

Antitox. Diph.—Purified Antidiphtheric Serum, Concentrated Diphtheria Antitoxin, Refined Diphtheria Antitoxin, Antidiphtheric Globulins

DIPHTHERIA Antitoxin is a sterile aqueous solution of antitoxic substances obtained from the blood serum or plasma of a healthy animal of the genus *Equus*, which has been immunized against diphtheria toxin. After the serum or plasma from the immunized animal has been collected, the antitoxin-bearing globulins are separated from the other constituents of the serum or plasma and dissolved in freshly distilled water. Sodium chloride and a preservative are then added and the solution is filtered through a bacteria-excluding filter. Diphtheria Antitoxin has a potency of not less than 500 antitoxic units per cc.

Description and Physical Properties

● A transparent or slightly opalescent liquid, of a faint brownish, yellowish, or greenish color, nearly odorless, or having an odor due to the presence of a preservative; it may have a slight granular deposit. Diphtheria Antitoxin must be free from toxins, and must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol, if either of these is used) and its total solids must not exceed 20 per cent.

Diphtheria Antitoxin must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The potency of the Antitoxin shall be expressed in antitoxic units, and the unit shall be that of the standard Diphtheria Antitoxin distributed by the National Institute of Health of the United States Public Health Service.

The outside label must bear the name *Diphtheria Antitoxin* and indicate the minimum number of antitoxic units in the package, the manufacturer's lot number of the antitoxin, the name, address and the license number of the manufacturer, and the date beyond which the minimum potency of contents, as declared on the label, may not be maintained. This date is one year from the date of issue from the manufacturing establishment if at that time the Antitoxin placed in the container had an excess of 20 per cent over the declared minimum potency, two years for a 30 per cent excess, three years for a 40 per cent excess, or four years for a 50 per cent excess.

Storage

● Preserve Diphtheria Antitoxin at a temperature between 2° and 10°C.,

preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● By parenteral injection, therapeutic, 10,000 units. Prophylactic, 1000 units."

HISTORY

THE diphtheria bacillus was first described by Klebs in 1883 and in 1884 it was isolated by Loeffler. Referred to as the Klebs-Loeffler bacillus, it is also the type organism of the genus *Corynebacterium.* Mucous surfaces are the favorite site for the growth of the diphtheria bacillus. The pharynx, larynx and nasal passages are the areas most frequently attacked. Envolvements of the mucous membranes of the eye, stomach and vulva and diphtheritic wound infections have been observed occasionally. In diphtheria, there may be a mechanical disturbance due to local inflammatory ulceration, but practically most of the clinical symptoms are the result of the absorption of the extracellular toxin produced by the bacillus. It is this toxin which determines the systemic characteristics of the disease and constitutes the most frequent cause of death.

Emil von Behring demonstrated that animals could be treated with diphtheria toxin prepared in the laboratory, with the result that the serum of the blood of animals could be made to yield large quantities of diphtheria antitoxin. This serum could then be used in the treatment of and as a temporary preventive remedy against diphtheria in human beings due to the specific neutralization of the toxin produced by the bacilli. In 1894, large scale production of diphtheria antitoxin was started by various commercial houses so as to make available this specific remedy for the scourge diphtheria. In December of the same year, the New York Herald antitoxin fund was turned over to the New York City Health Department. The following year, the latter bureau began to make, distribute and administer this remedy.

METHOD OF PRODUCTION

● The methods for producing diphtheria antitoxin commercially vary only in minor technical details. The first step for successful antitoxin production is obtaining a diphtheria culture of high-toxin producing capacity and growing it so as to yield a strong potent toxin. For the production and standardization of such toxin, see under Toxinum Diphthericum. For antitoxin production on a large scale, healthy horses have been found to serve as the most useful animals. The horses that are selected are moderately young, vigorous, of fair size and they must be absolutely free from disease. They are given a thorough examination by a veterinary surgeon and kept under close observation for at least two weeks before immunization. The tuberculin and mallein tests are performed to insure freedom from tuberculosis and glanders; and a prophylactic dose of tetanus antitoxin is given so as to create a temporary immunity against tetanus. No animal is retained unless found to be perfectly fit. Healthy horses possessing some natural antitoxin are preferred as in practice they have proven to be the animals which are capable of producing a large yield of antitoxin in their blood. Horses whose sera are found to contain 1/20 unit or more per cc. are always selected, when available. It is probable that the tissue cells of the naturally immune animals have acquired the property of giving a quick response to stimulation of diphtheria toxin.

The horses selected are each given a preliminary intravenous injection of 3000 units of diphtheria antitoxin on the day preceding the first injection of toxin. They are then injected subcutaneously with minute and then slowly increasing doses of the diphtheria toxin. Park advises as the first injection, ten M.L.D.'s of toxin diluted with 50 cc. of saline solution. Subsequent injections are given every second day, the increase of toxin injected being by 100 percent for the first seven injections, by 75 percent for the next seven injections and then by 50 percent for the next series. The rate of increase is then gradually lowered to 10 percent, which is maintained until the maximum antitoxin content for the animal is reached. Wadsworth has recommended an initial dose of 250 M.L.D.'s made up to 20 cc. with saline solution. Subcutaneous injections are given every third day until 18 injections (the last injection consisting of 30,000 M.L.D.'s of toxin) have been given. The schedule may vary when indicated by the reactions induced. Diphtheria toxoid is used now instead of toxin or toxin-antitoxin mixtures. It is employed in large initial doses (5 to 20 cc. for the first injection) and need not be preceded by diphtheria antitoxin. The horses become immune so that it becomes possible to inject larger doses of toxin, or toxoid, which if given in like amount at the beginning would have proven fatal. The antitoxin content in the blood of animals so injected reach a certain peak and it is not increased even though additional injections of toxin are given. A trial bleeding will reveal the antitoxin content. Immunization of horses whose serum contains less than 100 units of antitoxin per cc. after a period of two months is generally discontinued as such animals are unsuitable for diphtheria antitoxin production. If only high grade antitoxin is wanted, and this is most frequently the case in commercial production, all horses whose maximum yield is 300 units or less per cc. are discarded, and are not used for diphtheria antitoxin production. Most horses approach a yield of 300 to 500 units per cc. of serum within three months. Higher levels are not uncommon. A moderate percentage of horses will give 800 units per cc. of serum or better. Those exceeding 2000 to 2500 units are exceptional although Archipoff reported a horse whose serum yielded 3500 units per cc. The author knows of commercial laboratories producing horse serum at all times of 1500 to 2000 units per cc. or better. The horse having thus been brought up to a "production basis" is ready for bleedings. Trial bleedings are generally taken before the fourteenth and then before every fifth injection thereafter or oftener until whole or large bleedings are commenced (when the antitoxin content is sufficiently high or the peak has been reached). When large bleedings are started, two injections two days apart are given and blood is taken six or seven days later. These bleedings are generally continued until the antitoxin content of the serum approaches the minimal limit (250 units per cc.) or until the condition of the horse makes further immunization inadvisable.

Treatment (further injections with toxin or toxoid) is discontinued and the horse is bled out. Occasionally it may be found advisable to give a horse a rest (discontinue bleedings and immunization treatment). Depending upon the recuperative power of the animal, the length of the rest period may vary. On reimmunization, an equally potent serum as was yielded originally will be produced again. At each large or whole bleeding, nine liters of blood (from a horse weighing approximately 1200 pounds) are taken. When a horse is bled out, from 22 to 30 liters of blood are usually obtained.

Where toxoid alone is used for immunization, the doses in subsequent

injections are increased by 10 to 20 cc. until 200 cc. is reached. This is followed by 50 cc. of toxoid containing calcium chloride (each liter of toxoid containing 25 cc. of a sterile 33% calcium chloride solution). The maximum dose of toxoid plus calcium chloride is 200 cc. Several sites are used for the injection of large volumes.

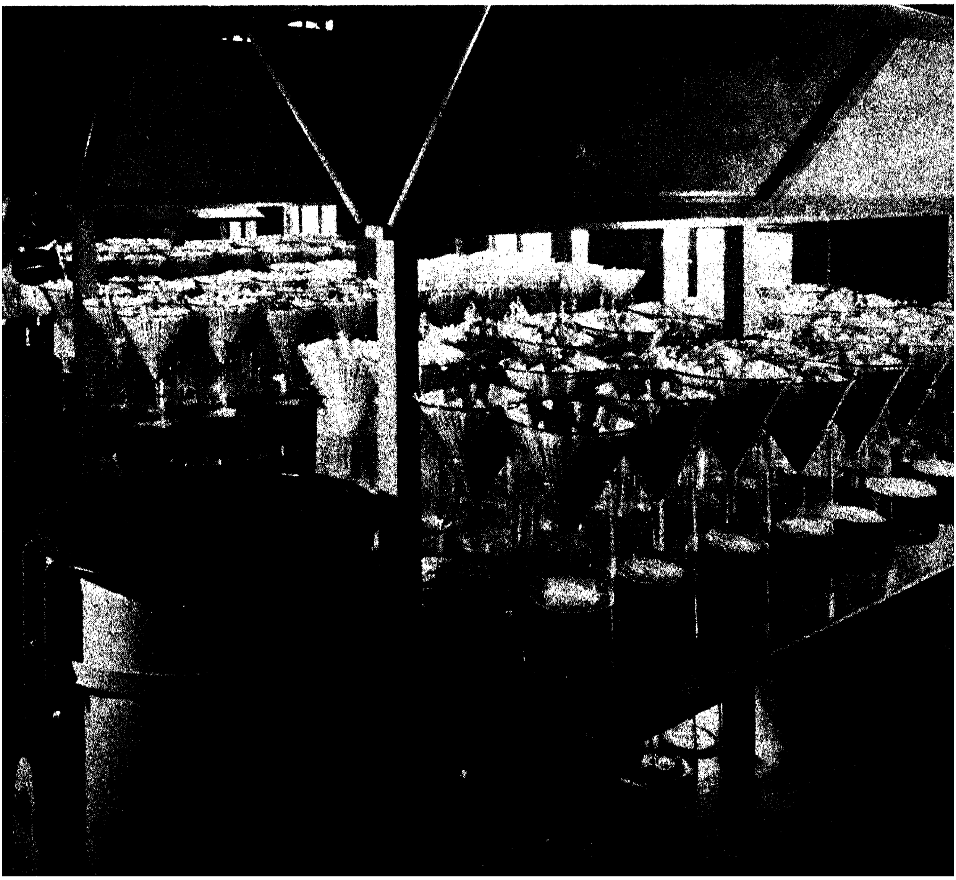
The horses to be bled are led into a specially constructed stall where the operation of bleeding is under perfect control. These stalls are in buildings of solid concrete and are separated from the stables and other buildings. All walls, floors, ceilings and compartments are constantly kept thoroughly aseptic. Apparatus, instruments and all equipment that are used here are thoroughly sterilized. The immunized animal is first cleansed well with soap and water, the neck is shaved, and asepsis is maintained by washing with an antiseptic solution and covering the entire body (except head and jugular vein) with a sterile sheet or apron. A sterile sharp-pointed cannula (to one end of which is attached a sterile rubber tube) is inserted into the jugular vein. The latter is at times made prominent by the use of a special tourniquet or by other means of compression, after the skin directly over and parallel with the vein is slightly incised (¼ to ¾" incision through the skin). The blood is allowed to flow through the rubber tube into the inlet tube of the high sterile glass cylinders or bleeding jars (placed on a low stool) and containing a 17 percent. sodium citrate solution in amounts equal to one-tenth of the volume of blood which is to be added. Ligature of the vein after bleeding is not necessary. This citrated blood is processed to separate the serum, for the latter contains the antitoxic substances or antitoxin.

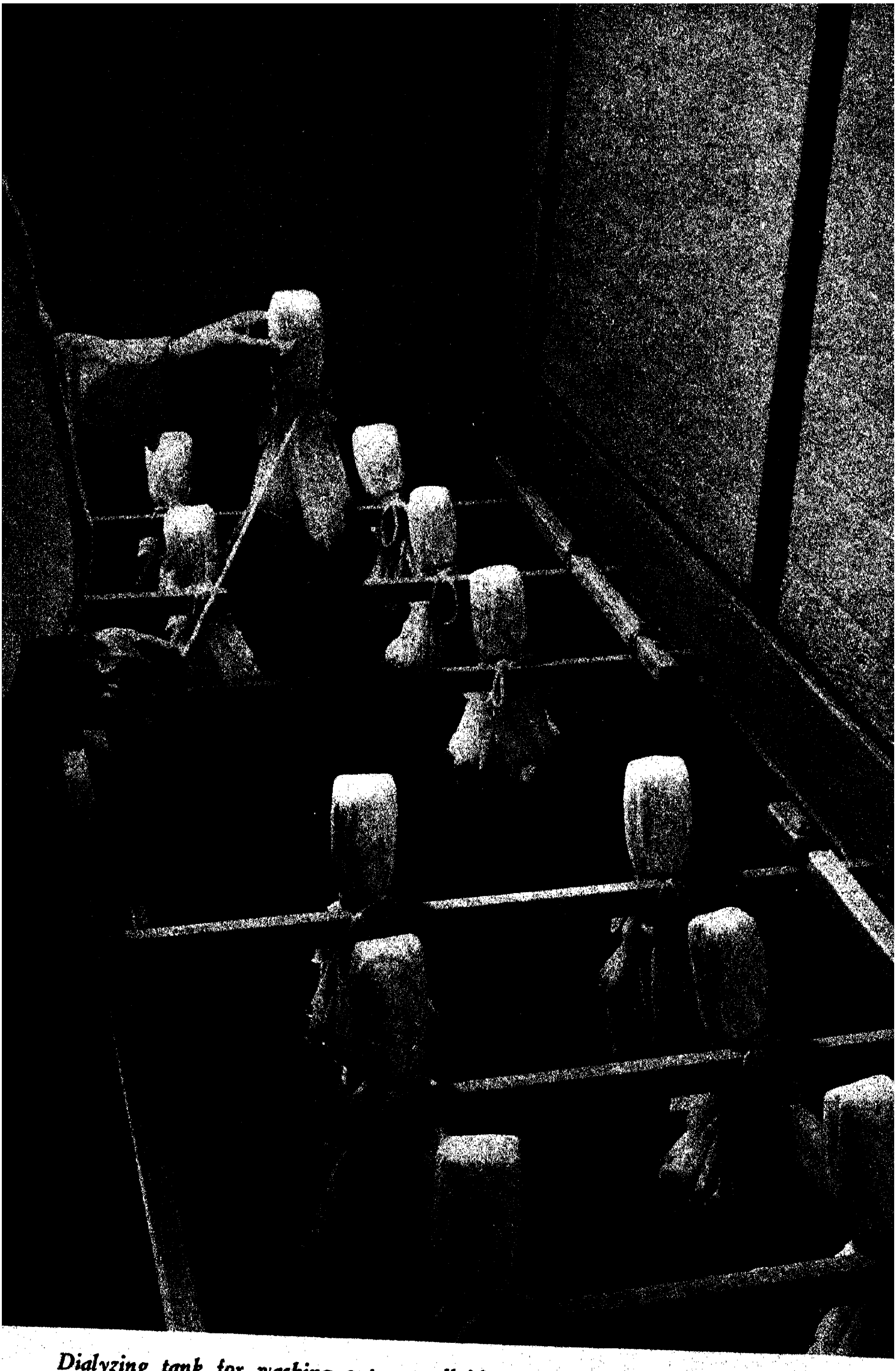
CONCENTRATED DIPHTHERIA ANTITOXIN

● By means of improved methods in the concentration and refinement of the antitoxic blood serum, it is possible to eliminate most of the undesirable albuminous substances and other valueless constituents of the serum. There is then available in a small volume a superior product of high concentration and potency as to its antitoxin value and low in its content of albumins and solids. The result is that it is easier to administer such a product; there is less pain to the patient and a quicker absorption is the result; and what is of greater importance is the fact that the rashes and other undesirable effects produced in the patient and caused by the injection of blood serum (due to the albumins present) are less apt to follow. The cylinders of blood and citrate (to which 0.3% tricresol may be added to prevent contamination) are kept below 10°C. This antitoxic serum contains pseudoglobulins, euglobulins, albumins, nucleoproteins, cholesterin, lecithin, bile pigments and salts, non-protein nitrogenous compounds, inorganic salts, and water. In 1906, Gibson (*J. Biol. Chem.*, *1*, 161 (1906); *3*, 233, 254 (1907)) announced a method for the concentration of diphtheria antitoxin. Later Banzhaf (*Dep't. of Health, N. Y. City*, *4*, 202, 225, 230 (1908-09); *7*, 114 (1912-13)) made important modifications of this technique. Additional researches have led to further improvement in concentration and refinement. The methods consist of obtaining the antitoxin-bearing pseudoglobulin free from euglobulin, serum albumin and other serum constituents. The following procedure is used in the Laboratories of the New York City Health Department:

To the citrated plasma containing 0.3% tricresol, add an equal volume of water. To 7 parts of this mixture add 3 parts of a saturated solution of

Collecting the pseudoglobulin by paper filtration during the precipitation method of concentrating antitoxins.





Dialyzing tank for washing out crystalloids during the precipitation method of concentrating antitoxins.

ammonium sulfate (30% saturation). Heat at 57° to 63°C. for one hour, and filter while hot. This yields two fractions:

A. Precipitate	*B. Filtrate*
(contains euglobulins, some pseudoglobulins and fibrinogen.	(contains pseudoglobulins and albumins.)
The precipitate is washed with 30% saturated ammonium sulfate solution.	Add washings from fraction A.
Dissolve in water; add NaCl to saturation (to dissolve out the pseudoglobulins); and filter.	Add sufficient saturated ammonium sulfate solution to raise concentration to 50% (pseudoglobulins precipitate); and filter.
Add preservative, tricresol, 0.4%. doglobulins) and filter.	Precipitate is pressed between pads to remove excess of ammonium sulfate solution.
Precipitate is pressed to remove excess water and acid.	Place in dialyzing bag and dialyze to remove salts.
Place precipitate in dialyzing bag and dialyze to remove salts.	Add preservative, tricresol 0.4%.
Add preservative, tricresol 0.4%.	

Combine A and B fractions and correct the pH to 5.4 with normal acetic acid. Add 4 volumes of water containing 0.2% tricresol. Allow to stand in a cool place until acid precipitate is well settled, then siphon off the supernatant liquid, filter through pulp, adjust the pH to 6.8 with normal sodium hydroxide volumetric solution, add an equal volume of saturated ammonium sulfate solution, filter, press precipitate, dialyze, add 1% sodium chloride solution and 0.4% tricresol or other suitable preservative, and filter through pulp and Berkefeld or other type of bacteria-excluding filter. Test for sterility, toxicity and potency; place in sterile containers; and keep at temperatures not exceeding 10°C. The marketable preparation is transparent or slightly opalescent, possessing at times a slight granular deposit, of a faint brownish, yellowish or greenish color. It should be preserved at a temperature between 2° and 10°C. preferably at the lower limit. At low temperatures, the annual rate of deterioration does not exceed 10 percent but at temperatures between 15° and 20°C., it may approach 20 percent per annum. The contents of the finished marketable diphtheria antitoxin are to possess a potency of not less than 500 units per cc. If the dried globulins are marketed, the potency is to be not less than 4000 units per gram of powder. The expiration date is one year after date of manufacture or date of issue, with a 20 percent excess of potency; 2 years with a 30 percent excess; 3 years with a 40 percent excess; or 4 years with a 50 percent excess.

PURIFICATION BY ENZYME DIGESTION

● Different enzymes as pepsin, papain and trypsin have been used for the refinement of diphtheria antitoxin. The process is based on a controlled method of selective digestion of the proteins of the immune horse serum with one or more of the previously mentioned enzymes, usually pepsin. Sandor (*Compt. rend. soc. biol., 130,* 849 (1939)) presents a technique of antitoxin purification by enzyme digestion in which a purified diphtheria antiserum almost 5 times richer in antibody than the original is obtained.

Hutchinson (*Brit. Med. J.*, *1*, 384 (1939)) gives results obtained in a group of 200 patients, treated with a diphtheria antitoxin refined by a process of enzyme digestion followed by differential heating. There was a reduction in the fatality rate as compared with cases treated with ordinary concentrated antitoxin and fewer reactions were recorded. Sandor (*Compt. rend. soc. biol. 130,* 1187 (1939)) also presented a technique for obtaining diphtheria antitoxin of a very high degree of purity by pepsin digestion of the specific toxin-antitoxin flocculate.

STANDARDIZATION OF DIPHTHERIA ANTITOXIN

● Not only are the establishments manufacturing diphtheria antitoxin and similar biological products shipped in interstate commerce licensed, their laboratories inspected and their products tested for purity by the U. S. Public Health Service so as to be assured of the purity of the marketed preparations, but in the case of diphtheria antitoxin these establishments must record the potency of the latter in terms of "antitoxin units" so as to conform with the uniform standard for measuring the strength furnished by the department at Washington. Perhaps as good a non-technical definition as any is that each unit of diphtheria antitoxin represents that amount of antitoxin which will neutralize 100 minimum lethal doses (M.L.D.'s) of freshly prepared diphtheria toxin (definition of M.L.D. of toxin mentioned under Diphtheria Toxin). The marketed diphtheria antitoxin contains not less than from 1000 to 2000 or more of such units in each cc. of contents, though the U.S.P. requirements are for not less than 500 units per cc. If in dried or solid form, diphtheria antitoxin must possess a potency of not less than 4000 units per gram.

UNIT OF DIPHTHERIA ANTITOXIN

● As mentioned above, antitoxin units are measured in terms of toxin. The unit of antitoxin originally established by Ehrlich as the minimum amount which completely neutralizes 100 M.L.D.'s of the specific toxin, is used as a standard. It is now spoken of as an *"Old Unit."* Uniformity of measurement and standardization necessitates the possession by the laboratories producing antitoxin of a standard or uniform toxin. Inasmuch as diphtheria antitoxin is a more stable product, uniformity of diphtheria toxin is obtained by means of a standard diphtheria antitoxin distributed by the National Institute of Health at Washington, D. C. Containers of the standard antitoxin distributed by the latter are sent to all licensed manufacturers and they are labelled as to the number of units of antitoxin present in each cc. of contents. The unit of diphtheria antitoxin established by the National Institute of Health is the amount of antitoxin which will exactly neutralize the so-called L_+ dose of diphtheria toxin when both are injected simultaneously (or mixed together, allowed to stand at room temperature, protected from light for 15 to 30 and not more than 60 minutes and then injected subcutaneously) into a 250-gram healthy guinea-pig.

The L_+ ("Limes plus limes death, Limes tod or limit of death") dose of a diphtheria toxin is the smallest amount of toxin which when mixed with one standard ("old") unit of diphtheria antitoxin and injected into will kill the average 250 gm. guinea-pig at the end of the fourth day. The Lo ("Limes zero" (Limes threshold or Limes nul)) dose of diphtheria toxin is the largest amount of toxin apparently completely neutralized by one unit of diphtheria antitoxin. Theoretically the Lo dose of diphtheria toxin should contain 100 M.L.D.'s and the L_+ dose should contain 101 M.L.D.'s. The difference between the two doses should be 1 M.L.D. Practically however the

difference is found to be much more. Theoretically the unit of diphtheria antitoxin should be that amount of antitoxin which when mixed with an L_+ dose of toxin and injected subcutaneously into a guinea-pig weighing 250 grams will preserve the life of the guinea-pig for only four days, (i.e. will kill on the fourth day). In commercial practice where a margin of safety is advantageous the unit of diphtheria antitoxin (frequently designated the "New Unit") is regarded as the smallest amount of antitoxin which will save the life of a guinea-pig if injected together with a L_+ dose of toxin.

STRENGTH

● The strength of a sample of marketable diphtheria antitoxin is determined by comparing the amount of it which is required to protect guinea-pigs against the effect of a definite and fixed amount of a test diphtheria toxin with the amount of a standard preparation of diphtheria antitoxin necessary to give the same protection. In all final tests of antitoxin standardization the standard diphtheria antitoxin distributed freshly by the National Institute of Health (in the United States and by the Medical Research Council in Great Britain) should be used as a control. In preliminary tests accurately standardized manufactured products (antitoxin and toxin) kept for standardization only may be substituted if necessary.

In preparing a test toxin for assay of samples of antitoxin the method of production is the same as given under diptheria toxin. It is then stored (toluene is used at times as a preservative) in the dark at a temperature between 0°C. and 5°C. After several months there will be a decrease in the toxicity, which will be revealed by an increase in the M.L.D. and the L_+ dose. When the L_+ dose becomes stabilized (shows no further change), the test toxin is suitable for use. One possessing an M.L.D. between 0.01 and 0.001 cc. and revealing a difference between its L_+ and Lo doses which does not exceed fifteen times the M.L.D. is satisfactory. Dilutions of the Federal standard antitoxin are mixed with varying quantities of the stablized toxin to be used as the standard toxin, the mixtures are allowed to stand at room temperature (away from light) for one-half and not more than one hour, and injections are made subcutaneously into guinea-pigs weighing 250 grams. Thus the L_+ dose of the toxin is definitely established. The quantity (L_+ dose) of toxin is now mixed with varying dilutions of the unknown antitoxin to be standardized. These mixtures are treated as above and then injected subcutaneously into 250-Gm. guinea-pigs.

From the dilutions of antitoxin mixed with the L_+ dose which will kill and those which save the lives of the injected guinea-pig in the given time, the strength of the unknown antitoxin in terms of units is thus determined. The volume of antitoxin in the mixture which causes the death of a guinea-pig on the fourth day contains one unit of antitoxin.

TESTING OF STANDARD AND COMMERCIAL DIPHTHERIA ANTITOXINS

● The official regulations for testing standard and commercial diphtheria antitoxins as issued by the National Institute of Health (1938) follow:

Standard Unit of Diphtheria Antitoxin

● **The antitoxin was prepared by a commercial laboratory under the direction of J. F. Anderson, M. D., as described in Bulletin Number 21 of the Hygienic Laboratory, U. S. Public Health Service, The Immunity Unit, etc., M. J. Rosenau, April, 1905, second edition, 1912. The horse serum was dried by bubbling a current of air through the unconcentrated serum at about 35°C., and with a partial vacuum of about 23 inches (ca 575 mm.). For preservation an amber glass U tube with two extension arms was used. The connecting arm was plugged with cotton making 2 compartments, into one side of which slightly more than 1 gm. of the powdered dried serum was placed and into the other about 1 gm. phosphorous pentoxid, P_2O_5, was placed. The tubes were sealed under negative pressure and placed in properly marked pasteboard containers. Originally kept at 5°C., the supply is now kept in the fur room of a fireproof storage warehouse where the temperature ranges from 20°—40°F., usually 25°—35°F., with daily cooling of the room. Quantities**

sufficient for about 2 years are withdrawn as required and kept in the cold storage room of the Hygienic Laboratory, under lock, and at about 12°F.

A chemical balance is kept under lock solely for use in the toxin and anti-toxin titrations. The weights have been checked against weights of the U. S. Bureau of Standards. All quantitative glassware is calibrated by the U. S. Bureau of Standards.

Weighing the Antitoxin

● A weighing bottle is placed on the left hand side of the balance and weighed to tenths of milligrams. Five to ten minutes are allowed for taking up moisture before the final tare is determined. A 1 gm. weight is added to the other weights in the right hand pan, the balance is adjusted and the needle allowed to swing to the left, just before placing the antitoxin in the weighing bottle.

Upon withdrawal from the Laboratory stock, the antitoxin is used only provided the P_2O_5 still shows undissolved crystals. With a small file a cut is made into the extension arm of the U tube on the same side as the antitoxin, thus eliminating danger of forcing the cotton into the antitoxin when the vacuum is broken. Then the cross arm is cut. At the instant of breaking the tube, the first cut being broken first, the time clock is started. The antitoxin is placed in the weighing bottle, by tapping it through the straight extension arm, until the balance swings with the pointer slightly to the right of the mid-point. In case a quantity too greatly in excess of 1 gram has been added, it is removed with a sterile platinum spatula so shaped as to enter the bottle freely.

Readings are begun at the furthest swing to the right, then to the left and then to the right. The time is taken at the left swing as the median of the time of the observation. Observations are repeated until the pointer is practically at rest. A minimum of 5 observations is necessary. The data included in the first three columns of the following table (i. e. to the left of the vertical line) are recorded at the time of observation and the figures of the latter four columns subsequently calculated. The true zero moves to the right because of hydration of the antitoxin from the atmosphere.

Table I

Time at left reading	Readings on scale		True Zero	Gain in divisions	Elapsed time in seconds	Rate in gain in divisions per minute.
	L.	R.				
2 m. 50 sec.	2.2	8.2 7.5	2.825	——	——	——
3 m. 40 sec.	0.1	7.1 6.9	3.45	0.525	50	0.63
5 m. 40 sec.	R. 2.	6.0 5.8	3.95	1.125	170	0.39
7 m. 40 sec.	3.5	5.4 5.2	4.4	1.575	210	0.45
12 min. ——	5.8	6.0 6.0	5.9	3.075	550	0.34

The true zero is obtained by figuring the average of the 2 right readings against the left reading. Thus in the first observation the average of the right readings is 7.85 from which is subtracted the left reading 2.2, giving 5.65, half of which is 2.825. If the left reading is to the right of the midpoint it is added instead of subtracted, as for example in the fourth observation:— $\frac{5.4 + 5.2}{2} = 5.3$ and $\frac{5.3 + 3.5}{2} = 4.4$, the true zero.

The elapsed time is figured for each period together with the total gain to the right in true zero of the pointer. Thus in the second period the increase from 2 m. 50 sec. to 3 m. 40 sec., is 50 sec. The gain in divisions in the same period is from the first true zero, 2.825, to the second, 3.45.

3.45 — 2.825 = 0.525. In order to estimate the gain per 60 seconds the following formula is established:

$$0.525 : 50 \text{ (sec.)} :: x : 60. \quad 60 \times 0.525 = 31.500 \text{ and } \frac{31.500}{50} = 0.63$$

divisions gained per minute in the first period.

For the fourth period the formula would read 1.575 : 210 : : x : 60. x = 0.45

The increments in divisions per minute are averaged thus:—

$0.63 + 0.39 + 0.45 + 0.34 = 1.81$ and $\frac{1.81}{4} = 0.45$ division average gain per minute.

2 min. 50 sec. had elapsed from the time of opening the U tube to the time of the first observation of true zero. This is 2.83 min. 2.83 x 0.45 = 1.2735 the gain in weight at first observation. The true zero at the first observation is 2.825 division to right of center. 2.825 — 1.273 = 1.552 which represents the position of pointer to right when the U tube was opened. Each division represents 0.00031 gm. 1.552 x 0.00031 = 0.00048 gm. Thus, assuming that the rate of gain was the same before weighing began as after, the amount of dried antitoxin taken was 1.00048 gm. In the solution of the antitoxin this extra amount is not figured. It is not necessary to figure anything less than 0.0009 gm., and if the error weight is as great as this, it is preferable to take a new tube of antitoxin.

Dissolving the Antitoxin

● All glassware is chemically clean and sterile. With a 25 cc. pipette, 50 cc. of 0.85% sodium chloride solution is placed in a one liter volumetric flask, care being taken to avoid wetting the neck of the flask. A large glass funnel covered by an inverted Petri dish is then placed in the neck of the flask. The powdered antitoxin is slowly dusted from the weighing bottle through the funnel into the flask, care being taken to avoid clumps, and dissolved in the salt solution. A few cc. salt solution from a 50 cc. cylinder graduate are placed in the weighing bottle and the remaining antitoxin dissolved. This is poured through the funnel and the process repeated until all the antitoxin in the weighing bottle and all that on the funnel are washed into the flask, and until 283 cc. salt solution are used. This, together with the 50 cc. salt solution already in the flask, makes 333 cc. salt solution. Then chemically pure neutral glycerol (Colgate, Price, or Shoemaker

and Busch) is poured in until a point is reached about 1 cm. below the mark on the neck of the flask. Mixing is accomplished by rotation of flask with hands about its neck. In a glass cylinder graduate are placed 15 cc. salt solution and 30 cc. glycerol, and the two are thoroughly mixed. Both flasks and cylinder are placed in a cold room at about 10°C.

The second day following, the 66% glycerol in salt solution in the cylinder graduate is added to the flask up to the 1 liter mark. The antitoxin solution is poured from the flask into a 1 liter brown glass bottle and back again into the flask, the process being repeated 6 times. Care is taken to lose none of the solution, the flask and bottle being so handled that the last drop returns into the respective container. The solution is finally kept in the brown glass bottle. The solution is of such strength that 1.0 cc. = 6 units standard diphtheria antitoxin. By diluting in the ratio of 1.0 cc. this solution + 5.0 cc. salt solution the result is a solution containing 1 unit antitoxin per cc.

Before making this final solution, the amount needed for the test is determined. If 20 units were required, the dilutions would be made so as to provide an excess. Thus in the ratio of 1 + 5, the solution would be 4.0 cc. original dilution plus 20.0 cc. salt solution. The 20.0 cc. salt solution is placed in an immunity flask. Slightly more than 4.0 cc. original solution is drawn into a washout capacity pipette. The excess is withdrawn by means of wiping the tip of the pipette with sterile gauze until the meniscus is drawn down to the proper position. The original solution is blown into the salt solution and the mixture agitated. Then slightly more than 4.0 cc. are drawn into the pipette and blown back into the flask, the process being repeated 6 times, care being taken not to immerse the tip of the pipette too deeply. The contents of pipette are finally blown out into the flask, but since the mixture in the pipette is now essentially the same as that in the flask, the pipette need not be completely drained.

Testing the Antitoxin

● Two toxins are used each of which is thoroughly aged and ripened. Toxin 1742 B has an L_+ dose of 0.18 cc. Toxin E has an L_+ dose of 0.13 cc. It is recommended that the dilution be such that this dose is contained in from 1.5 to 2.5 cc. In addition to the antitoxin to be tested the antitoxin tested at the last titration is also employed, both in quantities of 1 unit in 1.0 cc. Each toxin is tested against each antitoxin, and each dose of toxin is used for 4 animals. The toxin is used in the L_+ dose and also in doses 3 to 8% less than the L_+, and in doses 3 to 8% greater than the L_+ dose. Thus with toxin 1742 B, there are used in addition to the 0.18 cc. doses, also 0.17 cc. and 0.19 cc. With toxin E there are used in addition to 0.13 cc., also 0.125 cc. and 0.135 cc.

Bearing in mind that the necessary volume of diluted toxin for each dose must be between 1.5 cc. and 2.5 and that the truly desirable amount is in the neighborhood of 2.0 cc. the dilutions for toxin 1742 B may be figured as follows:—Dilution 1-10 = 1.8 cc. per L_+ dose. This is somewhat smaller than is ordinarily used. Dilution 1-15 = 2.7 cc. per L_+ dose, which is too great a quantity. Dilution 1-12 = 2.16 cc. per L_+ dose, which is satisfactory. For the other doses 0.17 × 12 = 2.04 cc., and 0.19 × 12 = 2.28 cc. Since these are equally above and below the L_+ dose of 0.18 cc., the total amount needed for 24 syringes would be figured on the basis of the L_+ dose. Thus 0.18 × 24 = 4.32 cc. undiluted toxin. A slight excess is required and the undiluted toxin is measured in quantities not less than 1.0 cc. Therefore 5.0 cc. undiluted toxin are added to 55.0 cc. salt solution. This mixture is made with a washout capacity pipette and immunity flask in the same manner as with the antitoxin.

With toxin E (L_+ = 0.13 cc.) it is found that dilution 1-10 = 1.3 cc. is too little and that 1-20 = 2.6 cc. is too much. Therefore, dilution 1-15 = 1.95 cc. is satisfactory. The other doses are 0.125 cc. and 0.135 cc. Thus 0.125 × 15 = 1.875 cc., or for practical purposes 1.88 cc., and 0.135 × 15 = 2.025 cc., or for practical purposes 2.03 cc. Here again 0.13 cc. is the mean quantity used for each of 24 syringes and 0.13 × 24 = 3.12 cc. The mixture is made by adding 4.0 cc. salt solution.

Filling the Syringes

● The syringes used are Rosenau syringes, without side arm, with sterile vaselined needle attached by a short length of heavy rubber tube. They are set up in 4 rows of 12 syringes each as in the table below and each tube numbered. The toxins are first placed in the syringes from delivery pipettes properly graduated on the neck. Then the antitoxins are added from 1.0 cc. delivery pipettes. As each 1.0 cc. antitoxin dilution is added the syringe is actively rotated between the palms of the hands so as to insure thorough mixing. The table follows:

Antitoxin 229—1.0 cc. in each syringe

Syringe No.	1	2	3	4	5	6	7	8	9	10	11	12
Toxin 1742B—	0.17	.17	.17	.17	.17	.18	.18	.18	.19	.19	.19	.19 cc.
Syringe No.	13	14	15	16	17	18	19	20	21	22	23	24
Toxin E—	0.125	.125	.125	.125	.13	.13	.13	.13	.135	.135	.135	.135cc.

Antitoxin 228—1.0 cc. in each syringe

Syringe No.	25	26	27	28	29	30	31	32	33	34	35	36
Toxin 1742B—	0.17	.17	.17	.17	.18	.18	.18	.18	.19	.19	.19	.19 cc.
Syringe No.	37	38	39	40	41	42	43	44	45	46	47	48
Toxin E—	0.125	.125	.125	.125	.13	.13	.13	.13	.135	.135	.135	.135 cc.

The injections are commenced 1 hour after the antitoxin has been placed in the first syringe, all syringes meanwhile having remained at room temperature.

The guinea pigs are as nearly as possible of a weight of 250 grams. Weights below 240 grams or above 280 grams are unsuitable. The animals are identified by cards indicating color distribution, sex and weight. The weights are distributed in the groups of 4 animals so that no group will have heavy animals as against another group with light animals. The abdominal surface is widely clipped with a fine pronged mechanical clipper, but no antiseptic wash is applied. The needle is inserted subcutaneously through its entire length, passing across the linea alba so as to prevent leakage after withdrawal. Following injection of the toxin antitoxin mixture, an amount of salt solution sufficient to bring the total volume to approximately 4 cc. is placed in the syringe and injected. After withdrawal of the needle the area is gently massaged.

The Results

● In order for the antitoxin to be acceptable, the 8 animals injected with the L_+ dose of toxin should die in approximately 4 days, those given the largest dose should die sooner, and those given the smallest dose should survive longer. Owing to the fact that living animals are employed and to other factors in a biological experiment, the results may not be absolutely clear cut, and judgment must be exercised in the interpretations. In case of difficulty, the employment of two toxins and of two samples of antitoxin will give the clue as to whether the fault lies in toxins or antitoxins, and re-titration can be directed accordingly.

COMMERCIAL DIPHTHERIA ANTITOXINS

General Features

● The principle purpose of these tests is to determine that the antitoxins contain the units claimed on the label, plus a sufficient excess above the labeled unitage to protect against loss by deterioration before the date of expiration. Examinations are also made of color, turbidity, sterility, and total solids.

All data concerning the specimens are placed upon form cards. The specimens and cards are arranged in alphabetical order according to the name of the manufacturer, and in order of serial number if there are more than one sample of the same unitage from the same manufacturer.

Measuring the Volume

● Before any determinations are made, a protocol is arranged similar to that which will be given subsequently. A package is checked against cards and protocol, and the syringe label similarly checked. The syringe is completely discharged into a sterile 10 cc. cylinder graduated in 0.1 cc. under complete aseptic precautions. The cylinder is marked with a glass pencil to identify it. If the amount exceed 10 cc., a 10-20 cc. immunity flask is employed. After all the packages are emptied, the cylinders are examined to be sure no bubbles adhere above the main volume. If so, they are drawn up into a drawn-out sterile glass pipette and discharged into a main volume. The volume is read and recorded on the protocol in hundredths of cubic centimeters. Usually the lower edge of the meniscus is clearly visible. If too many bubbles are present, the exact volume must be approximated. The record on the protocol is placed over the claimed unitage.

The color of the specimen is determined by comparison with color standards. The standards deteriorate and must be renewed frequently. The basic solution is a saturated solution of Bismarck brown in absolute alcohol. Six standards are made, each indicated by a letter as follows:

A = 10 per cent base in absolute alcohol
B = 10 " " A " " "
C = 7 " " A " " "
D = 3 " " A " " "
E = 1 " " A " " "
F = 0.1 " " A " " "

The color is indicated on the card as C, D, or by combinations such as DE, etc., and entered also in the book of records.

The turbidity is compared with standards made as follows: One gram precipitated Fuller's earth which passes a 200 mesh sieve, is suspended in 1 liter distilled water. This equals 1000 parts per million. This is diluted to yield for separate tubes, 50, 100, 150, 200, 300 and 400 parts per million. The antitoxin is compared with the standards and the record made on the card and in the record book. If turbidity be greater than is represented by 400 parts per million, the specimen is regarded as unsatisfactory.

The test for sterility is made by inoculating 0.2 and 0.8 cc. (5 and 20 drops) of each specimen in 30 cc. broth fermentation tubes, from a 1.0 cc. graduated pipette. If the total amount of specimen be between 1.5 and 2.0 cc., inoculations are made with 1 and 2 drops. If the amount be less than 1.5 cc., the sterility test cannot be made. The fermentation tubes are examined after 2, 3, 4, and 7 days incubation at 37° C.

Total solids

● These are determined by desiccation over a water bath and weighing the dry residue or by use of the refractometer. The total solids should not exceed 20 per cent.

Determination of Dilution

● This is done so that 1.0 cc. final dilution will represent 1 unit of antitoxin in accord with the claimed unitage. An additional dilution must be made so that 1.0 cc. will contain 1.0 unit, if the excess unitage present meet the standard required by the Hygienic Laboratory to allow for deterioration up to the expiration date. If, as in the first example in the following protocol, it is claimed that 4.16 cc. contain 5000 units, dividing 4.16 into 5000 shows that 1.0 cc. contains 1200 units. The protocol shows that there should be 30 per cent excess unitage. $0.30 \times 1200 = 360$, which added to $1200 = 1560$, the number of units per cc., if the required excess be present. This method is applied to all the samples. If the sample be less than 1.0 cc., the same method is applied, but before making the dilution called primary in the protocol a 1:10 dilution is made in the graduated cylinder. Thus if the cylinder contain only 0.95 cc. sample, the 1-10 dilution would require a total volume of 9.5 cc. 9.5 cc. minus 0.95 cc. equals 8.55 cc., the amount of salt solution to be added from a graduated delivery pipette to the cylinder.

The primary dilutions are figured with an eye to the secondary dilutions, it being desired that the latter, which are made in immunity flasks because of the fractions of cubic centimeters required, shall be in the neighborhood of 20 to 30 cc. Nevertheless, since immunity flasks of this size may not be available in sufficient number, an alternative secondary dilution is figured in larger volumes. Thus if the supply of 20-30 cc. flasks be insufficient it will be possible to use others of 30-40, 40-50, 50-60, and 60-70 cc. capacity. A typical protocol follows.

Typical Protocol

Identification No.	Volume and Claimed Units	Percentage extra units required	Units per cc.	Primary Dilution	Secondary Dilution	Alternative Secondary Dilution
A. 6468 S	4.16		1200	1 + 39	1 + 29	
	5000	30	1560		1 + 38	
B. 6311 J	8.79		2280	1 + 99	1 + 21.8	2 + 43.6
	2000	15	2610		1 + 25.1	2 + 50.2
C. 1819 I	1.17		850	1 + 29	1 + 27.3	2 + 54.6
	1000	10	940		1 + 30.3	2 + 60.6
D. 2747 C	3.26		1530	1 + 49	1 + 29.6	
	5000	15	1760		1 + 34.2	
E. 8623 A	5.64		1770	1 + 49	1 + 34.4	
	10000	25	2210		1 + 43.2	
E. 8623 A	10.13		1970	1 + 99	1 + 18.7	2 + 37.4
	20000	25	2460		1 + 23.6	2 + 47.2
F. 32615	1.25		800	1 + 39	1 + 19	2 + 38
	1000	5	840		1 + 20	2 + 40
F. 33069	4.34		1150	1 + 49	1 + 22	
	5000	5	1210		1 + 23.2	
G. 38613	3.37		1480	1 + 49	1 + 28.6	2 + 57.2
	5000	5	1560		1 + 30.2	2 + 60.4

It will be noted that not less than 1.0 cc. of sample is used in the primary dilution. The required amount of salt solution is pipetted into an ordinary flask. The antitoxin sample is drawn, in slight excess, into a 1.0 cc. washout capacity pipettee, drawn down to the mark by wiping the tip of the pipette with sterile gauze, delivered into the salt solution and washed out 6 times For the secondary dilution, salt solution is placed in immunity flasks in the required amounts with precautions against wetting the neck of the flask, and the primary dilution delivered and washed out from washout capacity pipettes of 1.0 cc. or 2.0 cc. size as indicated in the protocol

Filling the syringes

● Three syringes are set up for each sample. Into each of the syringes is placed the L_+ dose of toxin, one toxin only being employed. Into the first of the syringes is placed 1 unit antitoxin sample corresponding to the claimed unitage. Into each of the other syringes is placed 1 unit antitoxin according to the required excess. Four additional syringes are set up for the control animals. Each of these contain the L_+ dose of standard toxin and 1 unit standard diphtheria antitoxin. The syringes are rotated actively to insure mixture and allowed to stand for one hour at room temperature. The records, method of identifying the animals, distribution of weights of the animals, method of injection and subsequent observations are the same as in setting up the standard unit.

Results

● The dilution representing the antitoxin with its required excess of unitage in accord with expiration date, contains a smaller absolute quantity of antitoxin than the dilution which accords with the claimed unitage. Thus in the first example in the protocol, the dilution in accord with claimed unitage is 1-1200 and in accord with the required excess 1-1560. The second of these dilutions should have less protective effect than the first. The animals receiving this second dose should therefore die sooner than those receiving the first. If the excess unitage be in accord with requirements, this second dose should contain 1 unit antitoxin, and since the L_+ dose of toxin is used, the animals should die on about the fourth day. If this smaller actual dose of antitoxin protect until the fourth day, the larger actual dose should protect at least as long (usually longer), and if the difference between the two be such that the larger dose is sufficiently so, the animal receiving it may survive the experiment. Two animals receive the smaller dose of antitoxin and should survive at least as long as the controls, usually the fourth day. One animal receives the large dose and, depending upon the amount of excess, may die on the fourth day, or a later day, or survive. The four control animals with the standard antitoxin unit should die upon the fourth day. No animal of the test series should die before the controls. The preparation of commercial antitoxins and methods of marketing are such that the antitoxins usually titrate higher than the label unitage and show a greater excess than is required for the expiration date.

OTHER METHODS OF STANDARDIZATION

Flocculation Technique

●Ramon demonstrated that toxin and antitoxin could be made to precipitate each other in proportions which depended upon the relative strength of the two reacting substances, (*Compt. rend. soc. biol.*, *86*, 661, 711, 813 (1922)),

This so-called "flocculation reaction" employed as is detailed elsewhere for the standardization of toxin and toxoid is useful in determining the antitoxin values of a given serum (*J. Path. and Bact.*, *27*, 187 (1924)). This method is used by many in preliminary titrations before the final Ehrlich Method is employed to obtain the unitage of antitoxin preparations.

Intracutaneous Methods

● The principle of these methods depends upon the fact that when small amounts of diphtheria toxin are injected intracutaneously into the abdominal area of animals, small areas of necrosis result within 48 hours. When mixtures of toxin and antitoxin are injected, the free toxin is revealed by the appearance of necrosis. Guinea pigs were first employed as the test animal (*Z. Immunitatsforsch.*, *3*, 208, 344, 433 (1909)); (*J. Path. and Bact.*, *24*, 61 (1921)). Rabbits have replaced guinea pigs as the former are more sensitive to diphtheria toxin; and because of the larger available skin area, more tests can be performed on a single animal (*J. A. M. A.*, *82*, 1114 (1924)). Intracutaneous methods of determining toxin and antitoxin contents are especially useful in investigations in which the amounts are to be approximated in blood, milk and other body fluids where only small quantities are found.

SPEED AND DOSAGE

● Experimentation and practical clinical observations in diphtheria have revealed that as time between the development and absorption of the poison or toxin in the system and the injection of the antitoxin grows longer, rapidly increasing doses of antitoxin are necessary and if too much time has elapsed no amount of antitoxin will save. It is therefore apparent that *the earlier Diphtheria Antitoxin is administered the more certain and rapid is the effect.* It has also been found that antitoxin injected subcutaneously is but slowly absorbed. Antitoxin is therefore given intramuscularly in all cases (the gluteal region or the anterior part of the thigh offer suitable sites). In the more severe cases and those in which an insufficient first dose was given or administration was delayed, an additional injection of antitoxin is given intravenously. It is better to give a sufficient and large amount as the first injection than to give many small doses at varying intervals during the same day. The dosage of diphtheria antitoxin for treatment depends largely upon the severity of the case, its stage of development when seen, and the age of the patient. It may vary from as low as 5,000 to 10,000 unit, in mild cases to from 20,000 to 60,000 in severe cases. In mild and moderate cases, the injection is administered slowly intramuscularly. In laryngeal and severe cases, intravenous or both intramuscular and intravenous injections are given. In malignant cases, intravenous administration is practiced. When given intravenously the dosage is one-half the amounts stated above. The U.S.P. average dose is 10,000 units as a therapeutic agent and 1000 units as a prophylactic agent. Toxin derived from all known types of *C. diphtheriae* appear to be identical in response to diphtheria antitoxin.

The dosage recommended by Schick is employed by many workers as a prophylactic agent in exposed susceptible individuals, 25 units per pound of body weight; for treatment in mild and localized diphtheria, 50 units per pound of body weight; in severe and serious cases, 250 units per pound of body weight. The intramuscular route is employed for administration in mild cases and intravenous administration is used in severe cases.

In a comparative test of natural and concentrated antitoxin in the treat-

ment of malignant diphtheria, Peters (*Brit. Med. J.*, II, 344 (1938)) advocates natural instead of concentrated diphtheria antitoxin for intravenous injection due to the less frequency in the immediate appearance of severe reactions. Ramon (*Soc. med. Hopitaux proc.*, July 8, 1937) presents interesting observations on combined therapy of antiserum with anatoxin. Treatment in 120 cases of diphtheria consisted of the simultaneous administration in two different parts of the body of 0.1 cc. anatoxin and 10,000 to 60,000 units of antiserum, followed by injections of anatoxin (0.5, 1, 2 cc., etc.) at 5 day intervals. The immunity eventually built up in these patients was only slightly less than induced by anatoxin immunization alone.

VALUE OF DIPHTHERIA ANTITOXIN

● Though diphtheria antitoxin is most valuable for the treatment of diphtheria it may be employed as a temporary preventive remedy against this scourge. Individuals who have been exposed to diphtheria may be protected from the disease by the administration subcutaneously of 1000 units of antitoxin. This immunity and protection does not last longer than from two to four weeks so that if it is necessary to prolong the period of protection, injections are to be repeated at least once a month. Where a large number of children or individuals exposed to diphtheria are to be given immediate temporary protection, it is advisable to first determine by the Schick test whether they do or do not possess a natural immunity. All those who are Schick positive and are in danger of contracting the disease are to receive the prophylactic dose of diphtheria antitoxin.



Proper refrigeration is a necessity.

It cannot be too strongly emphasized the enormous difference in the prognosis of diphtheria if antitoxin is administered very early. The careful diagnostician guided by the clinical history and physical findings may suspect clinical diphtheria in a suspicious sore throat. Under such conditions diphtheria antitoxin should be administered at once, after first taking cultures. No harm has been done by the injection if the laboratory findings are negative and the clinical symptoms disappear, but if the bacteriological examination is positive, much time has been gained and possibly a life has been saved. Diphtheria was known for its highly contagious character and the fatality rate among attending physicians was exceptionally high. In spite of all available medical knowledge of days gone by and the best of attention, there was but little which could be done for cases of diphtheria and but few diseases caused such shudders of horror or presented such high mortality. Epidemics were reported in the northern colonies in 1742, 1755, 1775, and 1787.

Throughout the nineteenth century, great epidemics of diphtheria spread through populated areas especially the large cities. Such epidemics were found and reported in the larger communities and epidemic waves recurred at 5 and 10 year intervals. The death rate in days gone by ranged between 20 and 60 per 100,000 and during periods of epidemics reached well over the 200 figure. Children are the ones most defenseless against this disease and quite naturally this scourge has been particularly a school disease spreading among children when congregating in large numbers in any environment. With the general improvement in sanitation, and the increasing use of modern methods of control, there has been a prolonged continued decrease in the incidence of diphtheria. These measures and more adequate methods of treatment have resulted in a decreased mortality during the past five or six decades to ten and even twenty fold.

The death rates from diphtheria for the entire country during the past quarter of a century reveal a steady decline due to modern scientific weapons.

The number of diphtheria deaths per 100,000 of population in the United States were:

1910	1915	1920	1925	1930
21	16	15	8	2.5

ACTION

● The chief action of diphtheria antitoxin is to neutralize the diphtheria toxin. It does this by not only neutralizing all free toxin circulating in the body fluids but it neutralizes toxin which has in the early stages united with the tissue cells. A stronger affinity exists between diphtheria toxin and antitoxin than between diphtheria toxin and body cells so that during an early stage in the less firm union of the latter two components the toxin may be dissociated to some extent by the more attractive antitoxin. Virulent diphtheria bacilli resist phagocytosis, due probably to a leukotoxic action of the diphtheria toxin aided by the fact that the latter probably affects or neutralizes opsonin. Diphtheria antitoxin neutralizes this antiphagocytic action of diphtheria bacilli and enables the latter to be digested.

DIPHTHERIA ANTITOXIN, B.P.

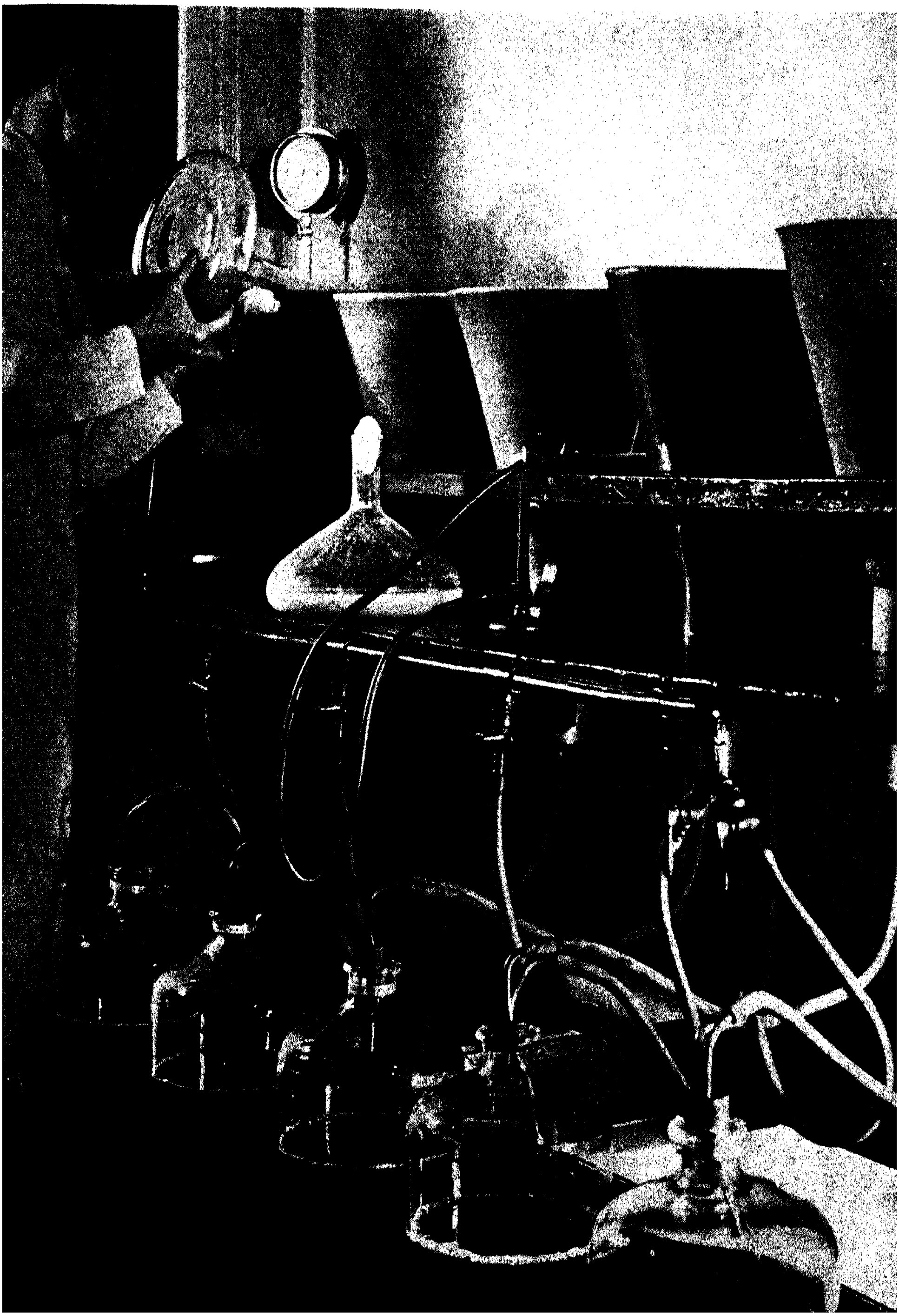
● In Antitoxinum Diphthericum, the U.S.P. recognizes only an aqueous solution of the antitoxic globulins. The Antitoxinum Diphthericum B.P. may be the whole serum containing the antitoxic substances in liquid form or dried or a preparation from the serum containing the antitoxic globulins (this preparation may be either in the liquid form or dried). Preservative is added to the liquid forms but not to the dried powder.

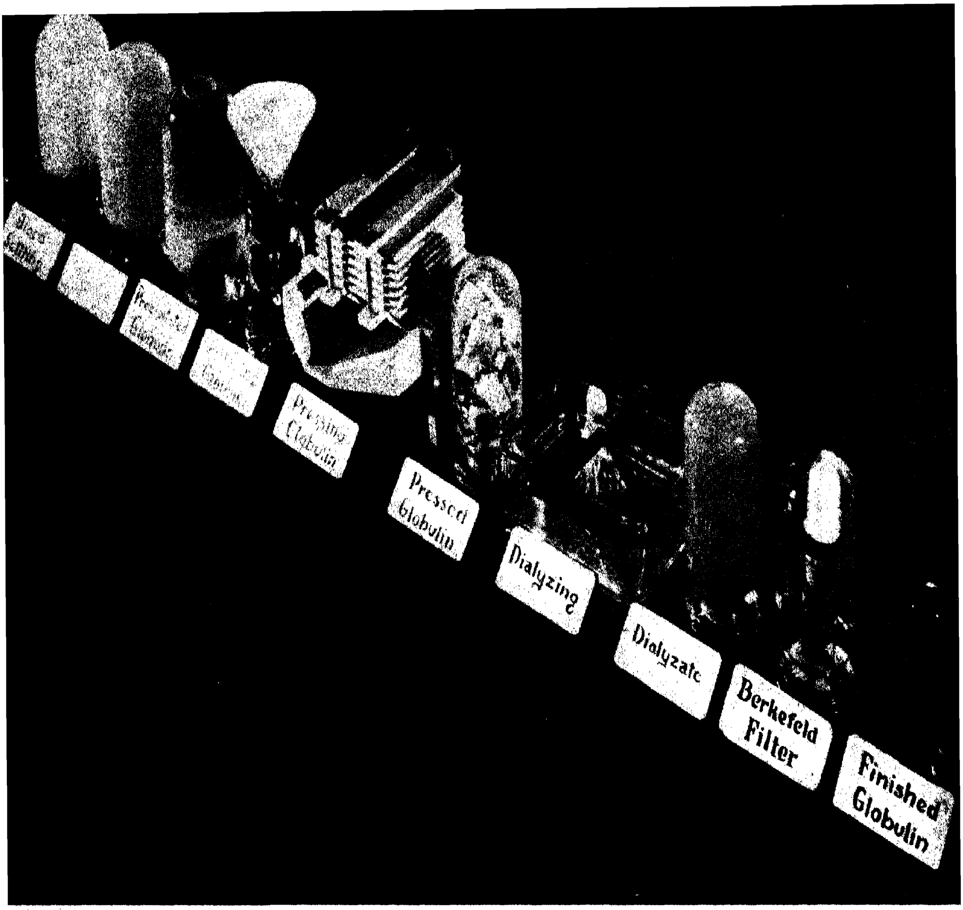
DIPHTHERIA ANTITOXIN, BOVINE

● This preparation differs from the U.S.P. diphtheria antitoxin in that it is made from the blood serum of immunized cattle instead of the blood serum of horses (equine). Individuals sensitive to horse serum receive this product for treatment when diphtheria antitoxin is needed.

The marketed bovine product is less potent than the U. S. P. equine preparation. The usual package is a sterile 30 cc. ampul vial containing 5,000 Units.

Candle filters separating diphtheria organisms from the diphtheria toxin.





Procedure showing individual steps of the precipitation method of concentrating antitoxins.

SERUM SICKNESS

● Although the use of concentrated and refined diphtheria antitoxic products has to a large degree eliminated the severe serum reactions, the latter may occasionally be encountered (in about one in each 10,000 persons injected). These reactions usually develop within a few minutes after injection and may be merely an immediate chill of a mild character or in more severe anaphylactic reactions, the face becomes flushed, respiration is accelerated, and other symptoms may appear as cyanosis, dyspnœa, and urticaria. Upon the appearance of the latter symptoms, 1 cc. of 1:1000 epinephrine should be given immediately. In few instances, death may result within a few minutes. Individuals who have not reacted badly to a first injection do not need to fear a second injection. So far as known all fatal results have followed the first injection. Mild reactions of serum sickness may occur from 6 to 20 days following the administration of antitoxin. The symptoms may be that of redness, pain or itching at the site of injection, pain in the joints, swelling of the lymph nodes, fever and a general urticaria lasting from a few hours to a week.

More recently, the use of histaminase has been used with encouraging results in the treatment of and as a prophylactic against serum sickness (*J.A.M.A.*, *112*, 1102, 2398 (1939)). If sensitization to horse serum is suspected or if the patient has a history of asthma, hay fever or a marked susceptibility to hives, .02 cc. of a 1:10 saline dilution of the antitoxin or of normal horse serum should be first injected *intradermally*. In a positive reaction, an urticarial wheal appears in 15 to 30 minutes at the site of the injection. The *ophthalmic test* for sensitivity to horse serum by instilling a drop of a 1:10 dilution of the latter into the conjunctival sac is used by many workers in preference to the intradermal test. A positive test is indicated within 15 minutes by conjunctival redness and swelling and increased lacrimation. Where the reaction is negative or where the history to sensitivity is negative, it is safe to proceed with the administration of the serum or serum product. If the patient is hypersensitive to horse serum and serum therapy must be employed, the patient should be desensitized. A suitable technique consists in administering subcutaneously the antitoxin in fractional doses beginning with 0.025 cc. If no reaction develops, double the dose every half hour until a total of one cc. was administered. Then inject intravenously 0.1 cc. diluted with saline solution. Double this dose every half hour until the full amount has been given. During desensitization a syringe containing epinephrine should always be at hand ready to use immediately.

Mention should be made here of the fact that other animals are used for the preparation of marketable products, the latter in turn to be employed for individuals sensitive to horse serum. Though fewer persons may be found sensitive to bovine serum, rabbit serum, or the serum of other animals as compared to horse serum, the same care must be observed with the former as when using the latter. Brown and Sechzer (*J. A. M. A.*, *111*, 1370 (1938)) present an interesting study on the sensitivity to rabbit serum and advocate the conjunctival test (with normal rabbit serum diluted 1:10) as a good indicator of general sensitivity.

IV

ANTITOXINUM TETANICUM

Tetanus Antitoxin

Antitox. Tet.—Purified Antitetanic Serum, Concentrated Tetanus Antitoxin, Refined Tetanus Antitoxin, Antitetanic Globulins

TETANUS ANTITOXIN is a sterile aqueous solution of antitoxic substances obtained from the blood serum or plasma of a healthy animal which has been immunized against tetanus toxin. After the serum or plasma from the immunized animal has been collected, the antitoxin-bearing globulins are separated from the other constituents of the serum or plasma and dissolved in freshly distilled water. Sodium chloride and a preservative are then added and the solution is filtered through a bacteria-excluding filter. Tetanus Antitoxin has a potency of not less than 300 antitoxic units per cc.

Description and Physical Properties

● A transparent or slightly opalescent liquid, of a faint brownish, yellowish, or greenish color, nearly odorless, or having an odor due to the presence of a preservative; it may have a slight granular deposit. Tetanus Antitoxin must be free from toxins, and must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol, if either of these is used) and its total solids must not exceed 20 per cent.

Tetanus Antitoxin must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The potency of the Antitoxin shall be expressed in antitoxic units, and the unit shall be that of the standard Tetanus Antitoxin distributed by the National Institute of Health of the United States Public Health Service.

The requirements for labeling are that the outside label must bear the name *Tetanus Antitoxin* and indicate the minimum number of antitoxic units in the package, the manufacturer's lot number of the Antitoxin, the name, address, and license number of the manufacturer, and the date beyond which the minimum potency of contents, as declared on the label, may not be maintained. This date is one year from the date of issue from the manufacturing establishment if at that time the Antitoxin placed in the container had an excess of 20 per cent over the declared minimum potency, two years for a 30 per cent excess, three years for a 40 per cent excess, or four years for a 50 per cent excess.

Tetanus Antitoxin prepared from the blood serum or plasma of an animal other than the genus *Equus* should be so labeled that the outside label also indicates the species of animal employed.

Storage

● Preserve Tetanus Antitoxin at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● By parenteral injection, Therapeutic, 20,000 units. Prophylactic, 1500 units."

HISTORY

LOCKJAW or tetanus has been recognized as a distinct clinical entity for many centuries. The disease is usually associated with a wound received from four to fourteen days previously. Though Nicolaier in 1884 produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden earth and described a bacillus which may have been the tetanus bacillus, it was Kitasato who in 1889 obtained the latter in pure culture, and described his method of obtaining it and its biological characteristics. In more recent classifications, the tetanus bacillus is grouped with the other large spore-bearing anaerobic bacilli in the genus Clostridium. It is therefore a frequent occurrence to find the tetanus bacillus mentioned as *Clostridium (C.) tetani.* The pathogenicity of the latter depends almost entirely upon the soluble toxins which it produces. The neutralization of these extracellular toxic agents by tetanus antitoxin becomes an important consideration in preventing this disease to develop, once the tetanus bacilli have gained entrance into the body.

PRODUCTION

● The production of tetanus antitoxin corresponds closely with those procedures described under the manufacture and standardization of diphtheria antitoxin which is to be reviewed for more detailed instructions not given in this text. The first step is to produce a potent tetanus toxin. Cultures of virulent tetanus bacilli retain their ability to produce toxin unaltered when kept under suitable conditions but if these cultures are subjected to deleterious influences they may lose this property. The toxin is produced *in vitro* by all strains of virulent tetanus bacilli, individual strains showing less variation in this respect than do individual strains of diphtheria bacilli. The yield of tetanus toxin in media varies according to the composition and reaction of the latter, the completeness of the exclusion of oxygen (as *Clostridium tetani* is an anaerobe), the age of culture, etc. Tetanus toxin is extremely sensitive and deteriorates rapidly in the presence of heat, light, oxygen, moisture and is readily affected by most chemicals. The medium most frequently employed for the production of tetanus toxin is a freshly prepared slightly alkaline (+1) beef-infusion bouillon containing 1% peptone (Berna), 0.5% salt, and 1% or 2% glucose. Under anaerobic conditions in the latter medium at 36° to 37°C. and with the exclusion of all light, the maximum toxin production is reached in from ten to 15 days (usually 15 days). After the twentieth day, the toxin begins to lose rapidly in strength. Cresol is added to the toxin broth cultures to give an approximately 0.25 percent solution, preliminary filtration is carried out through paper pulp and a coarse grained filter candle and then the final filtration is through a sterile Berkefeld filter. All glassware, filters, etc. should be neutral (to phenophthalein) and all light (direct or indirect) is excluded by darkening the room and covering all apparatus with dark cloths. Sterility and toxicity tests are conducted, the toxin being kept in the refrigerator excluding all light.

● Various procedures are practiced in different parts of the world for the standardization of tetanus toxin. In this country a unit of tetanus toxin is the smallest amount of toxin, which when injected subcutaneously, will kill a guinea-pig weighing 350 grams within ninety-six hours. This is the M.L.D. or M.F.D. of Tetanus Toxin. It may be of interest and of sufficient importance to note that the M.L.D. of tetanus toxin and the standardization of tetanus antitoxin in this country is measured against 350-gram guinea-pigs, while in European countries, it is measured either against guinea-pigs or against 20-gram mice. Freshly prepared toxin (with a fatal dose of 1/5000 cc. or a stronger toxin) is used for immunizing horses for the production of tetanus antitoxin. Relatively young horses of more than average weight are selected. Tests are not made for natural tetanus antitoxin content. In this country, the first two injections of toxin (consisting of 5000 and 10,000 units of toxin) are combined with 5000 units in the first and 1000 units of tetanus antitoxin in the second dose, the mixture of toxin and antitoxin being allowed to stand one hour before the subcutaneous injections are given. In Germany, a larger dose of toxin to which 0.25% of iodine terchloride is added is employed as the first injection. Injections are given every third day unless marked reactions develop. If the latter prevail, the next injection is given only after all symptoms subside. Tetanus toxin injections generally have a less deleterious effect upon the horse than is displayed by injections of diphtheria toxin. Tetanus anatoxin (formalinized toxoid) is used in France for the first immunizing injections. In America, the neutralizing antitoxin is eliminated after the second injection, and the increase of dosage of free toxin is by approximately 50 percent. This is also controlled by the determination of the antitoxin content of the animal's blood serum. Some methods add one-half percent alum to the toxin in the later injections. New methods of immunizing horses for the production of high titre tetanus and diphtheria antitoxin are receiving the attention of many workers. The use of antigen and alum (0.25 to 1%), antigen and tapioca (0.5%), followed later by antigen and calcium chloride (0.25%) or manganese chloride has been found to produce large yields of antitoxin (*Indian J. Med. Research, 25,* 617 (1938); 26, 311 (1938)). Tetanus anatoxin mixed with 1% tapioca and then with 1% calcium gluconate yielded a high-titre tetanus antitoxin in horses (*Indian J. Med. Research, 26,* 317 (1938)). If after from five to six months, less than 100 units of antitoxin are present per cc. of blood serum, immunization is discontinued and the horse is discarded for the production of tetanus antitoxin. Good horses yield a serum containing 200 to 600 units of antitoxin per cc. of blood serum and continue to produce serum of high potency for long periods. It is frequently found advisable to rest a horse producing large amounts of tetanus antitoxin for a few months at intervals of one or two years. When trial bleedings reveal that the titer of the serum has reached 300 or even 200 units per cc. of blood serum, whole bleedings (withdrawal of large amounts of blood, compatible with the health of the horse being bled) may be started. Immunization is generally continued until the titer falls below 150 units. The trial and whole bleedings, treatment of the blood, purification and concentration of the antitoxic material, testing for sterility and toxicity, etc., and marketing of the finished preparation are the same as in the case of diphtheria antitoxin.

UNIT

● The unit of tetanus antitoxin is different than that of the unit of diphtheria antitoxin. In the case of the tetanus, it was found that precipitated and dried tetanus toxin could be kept constant by special techniques of preservation so that tetanus antitoxin is standardized against a standard toxin (sent in the form of a dry powder in a sealed tube and supplied by the National Institute of Health at Washington). A standard tetanus antitoxin is also supplied for use in standardization and control tests. In this country, "*the unit of tetanus antitoxin is ten times the least amount of serum necessary to save the life of a 350-gram guinea-pig for ninety-six hours against the official test dose of standard toxin.*" The test dose consists of 100 M.L.D.'s of a precipitated toxin preserved under special conditions at the Hygienic Laboratories of the National Institute of Health. In other words, a unit of tetanus antitoxin is the least amount of antitoxin which will protect a 350-gm. guinea-pig from 1000 units or M.L.D.'s of tetanus toxin. Compared to a unit of diphtheria antitoxin tetanus antitoxin has slightly more than ten times the protective power (diphtheria antitoxin only neutralizes 100 units of diphtheria toxin and the latter is also weaker in toxicity than the tetanus toxin).

In the actual standardization of tetanus antitoxin, as in that of diphtheria antitoxin, the L_+ dose of toxin is employed. The L_+ dose of tetanus toxin which contains approximately 101 M.L.D.'s is defined in this case as the least amount of toxin which is capable of neutralizing 1/10 (American) unit of tetanus antitoxin and still contain a quantity of free tetanus toxin which would be sufficient to kill a 350-Gm. guinea-pig in ninety-six hours. It is of sufficient importance to note that the "American unit of Tetanus antitoxin" is twice as strong as the "International Standard Unit", the latter not having been adopted as yet by all of the nations represented at the 1926 Geneva Conference. The unit of Tetanus Antitoxin for Great Britain and Northern Ireland is one-half of the unit established in the United States. It is therefore of import to indicate in records and publications which of the unit standards were used.

Tetanus antitoxin must possess a potency of not less than 300 units per cc. if in liquid form, and not less than 2,400 units per gram if in solid form. Its deterioration is more rapid than is that of diphtheria antitoxin and the expiration date should be carefully noted. The latter is one year after date of manufacture or date of issue, with a 20 percent excess of potency; 2 years with a 30 percent excess; 3 years with a 40 percent excess; or four years with a 50 percent excess.

There is also marketed a *Tetanus Antitoxin (Bovine)*. The procedures in the production of this preparation are as given previously except that cattle are immunized against the toxin and the blood serum from the former is used to prepare the tetanus antitoxin. Its use is recommended in the immunization of certain types of individuals where the equine serum appears objectionable (where hypersensitiveness to horse serum exists and desensitization is inadvisable).

TESTING STANDARD AND COMMERCIAL TETANUS ANTITOXINS

The official regulations for testing standard and commercial tetanus antitoxin as issued by the National Institute of Health (1938) follow:

Standard Unit of Tetanus Antitoxin

In this case both toxin and antitoxin are kept in the dried state. The tetanus antitoxin is

dried and preserved in the same manner as diphtheria antitoxin except that the U tube contains slightly in excess of 0.75 gm. dried substance.

In preparing the dried toxin, the toxin broth is precipitated by super-saturation with ammonium sulphate. The creamy precipitate is collected on a watchglass, then placed on filter paper in a large Buchner funnel, over which a piece of rubber dam is loosely stretched. As negative pressure is applied to the filter flask the rubber dam exerts pressure upon the precipitate and aids in drying it. Upon removal from the funnel the mass is "worked" with a spatula, and then dried in partial vacuum over P_2O_5. The negative pressure must be just sufficient to "raise" the mass so that it will not "boil" over the sides of the dish. The P_2O_5 is replaced every two or three days until drying is complete. Any grossly visible crystals of ammonium sulphate are removed and the dry mass ground in a ball mill continuously for a week. In the case of the toxin now used, a mixture of two toxins was made in the ball mill. Thus it was possible to combine a strong toxin with a weaker toxin to obtain a mixture with the desired test dose of about 0.0006 gm. The resultant powder was placed in amber glass U tubes in quantities of about 1 gm. and P_2O_5 placed in the opposite arm. This is kept in the dark in a refrigerator. When a tube is opened and a small quantity removed for the test, the tube with the remaining toxin is kept in the dark in a high vacuum over H_2SO_4.

Weighing and Dissolving the Antitoxin

It is desired to weigh out 0.75 gm. tetanus antitoxin. This is done in exactly the same fashion as with diphtheria antitoxin. This quantity is dissolved in 1000 cc. 66 per cent glycerol in salt solution, in the same manner as with diphtheria antitoxin. The result is a solution which contains 5 units tetanus antitoxin per cc. The tests are made with 0.1 unit antitoxin instead of 1 unit. A dilution of 1-5 would give 1 unit per cc. and a dilution of 1-50 would give 0.1 unit per cc. Therefore, the dilution made for testing is by adding 1.0 cc. of the glycerol-salt solution mixture to 49 cc. salt solution.

Determination of the Quantity of Toxin Required

This determination is necessary before weighing and dissolving the dry toxin. It has been found that the test dose of the toxin now employed is 0.00057 gm. This is the amount of toxin, which when mixed with 0.1 unit antitoxin will kill the test animals in 4 days. It is necessary to dissolve and dilute the toxin so that the test dose, as with diphtheria toxin, will be contained in a volume between 1.5 and 2.5 cc. By diluting in the ratio of 1-4000, the volume of the original mass will be increased 4000 times. The error introduced by the fact that 0.00057 gm. toxin is not exactly 0.00057 cc. is not of significance. $4000 \times 0.00057 = 2.28$. Thus in a dilution of 1-4000, 0.00057 gm. will be contained in each 2.28 cc. This is within the desirable limits of volume. If 40 animals are to be used $40 \times 2.28 = 91.2$ cc. of the 1-4000 dilution which is required for the entire experiment. The primary solution of the toxin made in the weighing bottle is in the proportion of 1-100 and a subsequent dilution of 1-40 gives the required final dilution of 1-4000. The quantity of final dilution required is 91.2 cc., and a quantity slightly in excess of this is actually necessary. Beginning with the primary dilution, it is found that $1 + 39 = 40$ cc. is too little, as is also $2 + 78 = 80$ cc. $3 + 117 = 120$ is adequate. Because of limitations of glassware, this is set up as $2 + 78 = 80$ cc., and $1 + 39 = 40$ cc. Thus 3.0 cc. is the quantity of primary solution (1—100) of toxin necessary. To obtain this, not less than 0.030 gm. toxin must be weighed out. Since the primary solution must be drawn up into pipettes it is necessary to have a slight excess, and accordingly a slight excess over 0.030 gm. toxin must be placed in the weighing bottle.

Weighing the Toxin

The tare of the weighing bottle, with its stopper on the scale pan beside it, is determined. Then weights totalling 0.030 gm. are added to those in the opposite pan. The toxin is dusted into the weighing bottle until the pointer swings at about the mid-point. A slight additional amount of toxin is dusted in, the stopper placed in the weighing bottle and the exact amount of toxin determined. This is found to be 0.036 gm. Since the amount of dry material is so small and the stopper placed in the bottle so soon, it is unnecessary to determine the amount of water taken up by the dry product, an amount which is almost infinitesimally small.

Dissolving the Toxin

From this point onward, the window shades are drawn and the work done solely under illumination by carbon filament incandescent lamps. Since the weighing bottle contains 0.036 gm. dry toxin, it will be necessary to add 3.6 cc. salt solution to make the primary dilution of 1-100. This amount is slowly run into the weighing bottle from a graduated delivery pipette. Care must be taken not to cause the formation of large clumps of powder. It is desirable to run in the salt solution in such fashion that the powder will float on the surface. The bottle is gently agitated until all the dry toxin goes into solution, especial attention being given to masses which form on the bottom and which may have a protective surface of air. With a washout capacity pipette, 2 cc. of the primary dilution are withdrawn, to a point slightly above the mark, the column being brought down to the mark by wiping the end of the pipette with sterile gauze. The 2.0 cc. are then run into 78.0 cc. salt solution in an immunity flask, and washed back and forth 6 times, and finally blown out of the pipette. The same process is effected with 1.0 cc. primary solution and 39.0 cc. salt solution. The two final dilutions thus made are added together in a flask, giving about 120 cc. of a 1-4000 dilution of toxin, of which 2.28 cc. represents the dose of toxin—0.00057 gm.

Doses of toxin of three different sizes are employed as with diphtheria toxin, 5 per cent above and 5 per cent below that test dose described above. 5 per cent of 0.00057 gm. is 0.0000285 gm. For simplification this is regarded as 0.00003 gm. Then $0.00057 + 0.00003 = 0.00060$, and $0.00057 - 0.00003 = 0.00054$. Thus in addition to the test dose 0.00057, doses of 0.00060 and 0.00054 are employed. To determine how much of the 1-4000 dilution of toxin is to be employed in each instance, each figure is multiplied by 4000. Thus $0.00054 \times 4000 = 2.16$ cc., and $0.00060 \times 4000 = 2.40$ cc.

Filling the Syringes

This is done in exactly the same manner as with diphtheria toxin and antitoxin. One toxin, the last tested antitoxin and the newly dissolved antitoxin are employed as in the following

protocol. The toxin doses are given in milligrams.

Last antitoxin—1.0 cc. in each syringe												
Syringe No.	1	2	3	4	5	6	7	8	9	10	11	12
Toxin	.54	.54	.54	.54	.57	.57	.57	.57	.60	.60	.60	.60

New antitoxin—1.0 cc. in each syringe												
Syringe No.	13	14	15	16	17	18	19	20	21	22	23	24
Toxin	.54	.54	.54	.54	.57	.57	.57	.57	.60	.60	.60	.60

The Injections

These are commenced 1 hour after the antitoxin has been added to the toxin in the first syringe, all syringes meanwhile having remained at room temperature. The selection, distribution and identification of animals and all other precautions are the same as for diphtheria antitoxin.

The Results

In order for the antitoxin to be acceptable, the 8 animals given the test dose of 0.00057 gm. toxin should die in approximately 4 days, those given the largest dose should die sooner and those given the smallest dose should survive longer. Since autopsies will yield no information as to death from tetanus, it is essential to make observations of the animals on the second, third and fourth days for the presence of tetanic symptoms. These may be observed on inspection or it may be necessary to pick up the animal in order to elicit them. The symptoms are recorded in 5 degrees:— 1, hypertonicity, which is demonstrated by picking up the animal, 2, slight symptoms, visible to the eyes on handling, 3, symptoms, visible without touching the animal, 4, marked symptoms, including definite spasm but with the animal still on its feet, 5, severe symptoms, with practically continuous spasm and inability to keep on its feet. Even the animals with the smallest dose of toxin will show symptoms and die later than the others. The observations give reasonable assurance that those which die, do so because of tetanus. If they have shown no symptoms of the disease, death cannot be ascribed to it. The same careful judgment and evaluation of results are required here as with diphtheria tests.

COMMERCIAL TETANUS ANTITOXINS

General Features

The purposes of the test, the protocols, as well as the volume color turbidity, sterility and total solids of the samples are the same as for diphtheria antitoxin. Two differences are to be noted. The mixing of toxin and antitoxin is done with illumination by carbon filament incandescent lamps. The dilutions are carried one decimal further than with diphtheria antitoxins, because all the tests with tetanus are in tenths of a unit.

Determination of the Dilution

This is done so that 1.0 cc. final dilution will represent 0.1 unit of antitoxin in accord with the claimed unitage. The excess unitage required for expiration date is in the same percentage as with diphtheria antitoxins, and all other features of dilution are the same. A protocol follows. In this instance a toxin was used whose dose was 0.00066 gm., and controls set up with standard antitoxin.

TYPICAL PROTOCOL

Identification Number	Volume and Claimed Units	Percentage extra units required	Tenth Units per cc.	Primary Dilution	Secondary Dilution	Tertiary Dilution	Alternative Tertiary Dilution
1 55 H	2.82 / 1500		5320	1 + 99	1 + 52.2	——	——
1397 N		10	5860		1 + 57.6	——	——
M-5721	2.5 / 1500	10	6000	1 + 99	2 + 18	1 + 19	2 + 38
13 A			6600			1 + 21	2 + 42
N 425	2.27 / 1500	—	6610	1 + 29	2 + 18	1 + 21	2 + 42
P. 0827	2.5 / 3000	25	12000	1 + 29	2 + 38	1 + 19	2 + 38
89 C			15000			1 + 24	2 + 48
U. T53	2.45 / 1500	20	6120	1 + 29	1 + 60.2		
B 1.			7350		1 + 72.5		

It will be noted in the protocol that in order to arrange the unitage in tenths of a unit, the figure of units per cc. is determined and a zero added. On account of the limitations of glassware (maximum of 100 cc.) a tertiary dilution is sometimes necessary. To conserve pipettes it is sometimes necessary to make an alternative tertiary dilution; when this is shown on the protocol it is the dilution employed.

Filling the Syringes

These are filled in the same manner as with diphtheria toxin, but with the illumination by carbon filament lamp. The syringes stand in this light for one hour before injections are made.

The Results

The animals are observed for the five degrees of symptoms enumerated with the tests for standard tetanus antitoxin. The potency of the samples is evaluated in the same way as with diphtheria antitoxin, except that death is not ascribed to tetanus unless the animal has exhibited symptoms.

CAUSE OF TETANUS

● Tetanus (lockjaw) is caused by the tetanus bacillus (*Clostridium tetani*) which is present in the superficial layers of the soil. It occurs especially in cultivated and manured fields, probably because its normal habitat is in the dejecta of some of the domestic animals (cows, horses, etc.). It has been found in hay dust, in the dust of rooms, barracks and hospitals, and occasionally in courtplaster and catgut. On the skin surface and exposed to the air, tetanus bacilli and their spores are harmless. Entering the deeper tissues, as in puncture wounds and where anaerobiosis prevails, the spores develop into the vegetative forms and the latter proliferate producing toxin.

Considering the wide distribution of the bacilli around stables, in fertilized soils, in Nature, tetanus infections are of rather infrequent occurrence. The nature of the wound and the apparent simultaneous presence of other micro-organisms appear to be important factors resulting in the proliferation of tetanus bacilli. Injuries causing crushing and considerable destruction of tissues, especially deep, lacerated wounds (as frequently occur in compound fractures and gun-shot wounds) accompanied by contamination as result when grains of dust, chips or splinters of glass, metal or wood are embedded are most likely to be followed by tetanus infection. Apart from its occurrence following trauma, tetanus has been not infrequently observed after childbirth and in rare cases it has developed from the infection of necrotic mucous membranes following diphtheria and ulcerative lesions of the throat. At times very little local evidence of the disease can be observed and it should be borne in mind that when an external wound is not visible there may be an internal one. Regardless of the severity of the infection, a definite period of incubation elapses between the time of infection with *Clostridium tetani* and the appearance of the first symptoms, and in man, this may be from three or four to ten to fourteen days in acute cases to from four to five weeks in the so-called chronic form of tetanus. It is during this incubation period that the bacilli are multiplying and producing the toxin.

The pathogenicity of *Clostridium tetani* (tetanus bacillus) depends entirely upon the action of this powerful soluble toxin. Tetanus bacilli are localized almost entirely at the point of inoculation but the local infection is accompanied and characterized by a general toxinemia. Two toxins in reality are produced. These are named according to their action, the tetanolysin (produces some lysis of erythrocytes) and tetanospasmin (production of toxic spasms). Only the latter is of importance. It is one of the most powerful poisons known and human beings are extremely susceptible to its action. The harmful effects of this toxin are generally attributed to its affinity for the central nervous system where there is a

union of toxin with the gray matter of the brain and spinal cord. Once tetanus toxin is firmly united with nerve cells, it is difficult and practically impossible for tetanus antitoxin to effect its neutralization.

USE AS A PROPHYLACTIC AGENT

● Behring and Kitasato discovered tetanus antitoxin in 1892 and were the first to show the value of this preparation. In man, the most important use of tetanus antitoxin is as a prophylactic agent to prevent tetanus. The antitoxin will neutralize free toxin as quickly as it is secreted and before it is absorbed by the nervous tissue; and it may dissociate and neutralize some of the toxin "loosely united" with nerve cells. It should be borne in mind that the antitoxic effect passes off in about ten days and that only one immunizing injection of tetanus antitoxin may not always be sufficient. In the World War the compulsory routine use of tetanus antitoxin injections to every wounded soldier at the first possible moment after an injury and the administration of a second injection ten days later resulted in a marked reduction in the incidence and the almost complete absence of tetanus among the wounded troops. This extensive use demonstrated fully the value of tetanus antitoxin as a preventive agent (*J. Hyg.*, *19*, 1 (1920); (*Amer. J. Med. Sci.*, *151*, 668 (1916)).

It may not be possible to give set rules for prophylactic treatment. The suggestions and principles involved are that an injection of tetanus antitoxin is indicated in case of blank cartridge or other wounds where there has been laceration or crushing of tissue or puncture from rusty nails, bullets, needles, splinters and especially if dirt or soil has penetrated. A prophylactic dose of 1500 units of antitoxin should be given subcutaneously as soon as possible after the occurrence of the wound. In crushed wounds or those containing necrotic tissue or foreign bodies, a second dose should be given at the end of a week. If in rare cases the wound has not healed sufficiently, weekly injections of 1500 units of antitoxin are to be employed until the wound is practically healed. Whenever extensive injury occurs, larger doses, 3000 or more units are to be given for the first two doses.

USE IN TREATMENT

● Tetanus, once developed, is difficult and usually impossible to cure. Tetanus antitoxin in the treatment of developed tetanus does not appear to be of any distinct aid. Further investigation is needed to determine definitely whether this product possesses any definite curative value. When used for curative treatment, it is of vital importance that large doses of antitoxin are given at the earliest possible moment. As advised by Park and Nicoll (*J. A. M. A.*, *63*:235; *76*:112) the first injection should be made into the spinal canal. A lumbar puncture is made and a moderate amount of spinal fluid is withdrawn. From 5,000 to 10,000 units of antitoxin (warmed to body temperature) are then administered slowly by gravity (dilute if necessary with saline so that a volume of at least 5 cc. is injected). At the same time, 10,000 to 15,000 units are given intravenously. The intraspinal injection may be repeated in 24 and in 48 hours. Intramuscular injections of 5,000 units are given on the second, third and fourth days. If intraspinal injections are impossible, an intravenous dose of 15,000 to 20,000 units should be given. If intravenous therapy is impractical, 30,000 units of antitoxin are given intramuscularly, several muscles being used. Children need almost as much antitoxin as adults. Intraspinal and intravenous medication should be tried and injections given at the earli-

est possible moment. Recent investigations have demonstrated that large doses are needed and that intravenous administration must be carried to the limit. Studies on human subjects indicate it is not necessary to administer antitoxins intraspinally so as to reach the cerebrospinal fluid (*Rev. Immunol., 5,* 241 (1939)). Yodh (*Brit. Med. Jour.,* 1932, *II,* 589) has summarized an analysis of 278 cases of tetanus treated by various methods. Vener (*J. A. M. A., 110,* 2024 (1938)) administers a total dosage of 200,000 units (exclusive of desensitizing doses). He does not believe it necessary to continue serum therapy as long as the patient has spasms due to the persistence of the antitoxin in the blood stream.

Intrathecal administration of antitoxin is not advocated. Serum treatment in developed tetanus need not be continued for longer than 4 or 5 days or after a total of 200,000 units have been given. Any benefit obtained from antitoxin in the treatment of tetanus is probably due largely to the neutralization of uncombined toxin present in the blood stream, lymphatics and in the lesions from where it is absorbed.

A preliminary test for the detection of suspicious individuals sensitive to serum proteins and a technique of desensitization were described previously under "Diphtheria Antitoxin". The use of tetanus antitoxin does not preclude the employment of surgical treatment and other measures. The latter include: Early excision of the wound area, thoroughly cleansing the wound and removing foreign bodies, local applications of tincture of iodine, sedatives of the chloral type, heart stimulants when indicated, protection against excessive light, draught, noise and other annoyances and administration of large amounts of water (as the tetanus toxin is eliminated by the kidneys).

OTHER TETANUS ANTITOXIN PRODUCTS

● Ramon has prepared an "Antitoxin Solution" by making a 5% dilution of hyperimmune serum with saline, treating this with formalin and heating the mixture for one hour at 55° C. The finished preparation contains antitoxin units equivalent to the marketed tetanus antiserum and containing 20 times less the amount of protein. Reactions were infrequent (*Soc. med. Hopitaux proc.,* April 21, 1939, through *Presse med., 47,* 638 (1939)).

Goldie and Sandor (*Compt. rend. soc. biol., 130,* 1530 (1939)) have succeeded in attenuating or removing the anaphylactic activity of tetanus antiserum by treatment with ketene.

CHEMOTHERAPEUTIC AGENTS IN TETANUS

● Recent experimental studies on mice reveal protective properties of sulfanilamide or sulfapyridine against tetanus. In vitro experiments indicate that these drugs possess a neutralizing action upon tetanus toxin, the latter being oxidized to hydroxylamines or related substances (*Compt. rend. soc. biol., 130,* 1560-63 (1939)). It is possible that this may open a new field in the prevention and perhaps in the treatment of tetanus.

V

ANTITOXINUM SCARLATINAE STREPTOCOCCICUM

Scarlet Fever Antitoxin

Antitox. Scarlat. Streptococ.—Scarlet Fever Streptococcus Antitoxin, Refined Scarlet Fever Antitoxin, Concentrated Scarlet Fever Antitoxin, Anti-Scarlet Fever Globulins

"SCARLET Fever Antitoxin is a sterile aqueous solution of antitoxic substances obtained from the blood serum or plasma of a healthy animal of the genus *Equus* which has been immunized against the toxin or toxin-like substance produced by the streptococcus regarded as causative of scarlet fever. After the serum or plasma from the immunized animal has been collected, the antitoxin-bearing globulins are separated from the other constituents of the serum or plasma and dissolved in freshly distilled water. Sodium chloride and a preservative are then added and the solution is filtered through a bacteria-excluding filter. Scarlet Fever Antitoxin has a potency of not less than 400 antitoxic units per cc.

Description and Physical Properties

● A transparent or slightly opalescent liquid, of a faint brownish, yellowish or greenish color, nearly odorless or having an odor due to the presence of a preservative; it may have a slight, granular deposit. Scarlet Fever Antitoxin must be free from toxins, and must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol, if either of these is used) and its total solids must not exceed 20 per cent.

Scarlet Fever Antitoxin must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The potency of the Antitoxin shall be expressed in antitoxic units and the unit shall be that of the standard Scarlet Fever Antitoxin distributed by the National Institute of Health of the United States Public Health Service.

The outside label must bear the name *Scarlet Fever Antitoxin* and must indicate the minimum number of antitoxic units in the package, the manufacturer's lot number of the Antitoxin, the name, address, and license number of the manufacturer, and the date beyond which the minimum potency of contents, as declared on the label, may not be maintained. This date is one year from the date of issue from the manufacturing establishment if at that time the Antitoxin placed in the container had an excess of 20 per cent over the declared minimum potency, two years for a 30 per cent excess, three years for a 40 per cent excess, or four years for a 50 per cent excess.

Storage

● Preserve Scarlet Fever Antitoxin at a temperature between 2° and 10°

C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● By parenteral injection, Therapeutic, 6000 units. Prophylactic, 2000 units."

HISTORY

IT has been known for a long period of time that certain hemolytic streptococci produce certain soluble toxic substances. In an extended series of investigations by many workers, culminating in the investigations of Dick and Dick, (*J. A. M. A.*, *81*, 1166 (1923); *82*, 265 (1924)), evidence was presented that there existed a close relationship between the streptococcus and scarlet fever; and that hemolytic streptococci isolated from scarlet fever cases elaborated a rash-producing exotoxin. Recently Broadhurst brought to the forefront evidence that scarlet fever is in reality caused by a virus, a belief held by some workers even before the presentation of the work of Dick and Dick. There are some scientists who even hold that both a hemolytic streptococcus and a virus acting together are responsible for scarlet fever.

At present, however, the streptococcus theory is widely held and the toxin isolated from streptococci found in scarlet fever cases is used as a diagnostic agent (in the 'Dick Test') and for the preparation of products employed as a prophylactic against and in the treatment of scarlet fever.

Scarlet Fever Streptococcus Antitoxin consists of the solution of globulins obtained from the serum of horses which have been immunized against either (a) the soluble toxin of scarlet fever hemolytic streptococcus, after the method of Dick, or (b) by the injection of living streptococci employing the method of Dochez. In the latter procedure, horses are immunized by injecting subcutaneously small amounts of melted nutrient agar infected with the living organisms and (c) by a modification of either of these methods. In Dochez's method, the living streptococci are localized in the subcutaneous agar nodule, grow, and produce toxin *in vivo* and stimulate the production of antibacterial as well as antitoxic substances. The Dick method produces only an antitoxic product. The animals are immunized in the usual manner as employed for producing other antitoxins.

ANTITOXIN

● Scarlet Fever Streptococcus Antitoxin consists of the solution of globulin obtained from the serum of horses which have been immunized in the usual manner as employed for producing other antitoxins. When the serum has reached the maximum titer as revealed by standardization tests after trial bleedings, the blood is collected, concentrated and treated by methods similar to that used for diptheria and tetanus antitoxins, (with some light modifications). Scarlet Fever Antitoxin is a pale, yellowish gray liquid possessing the odor of the added preservative, and it is standardized by comparing its power of neutralizing scarlet fever streptococcus toxin with that of a standard serum supplied by the National Institute of Health. Its strength is expressed in terms of units. A unit (as the term is used here) is the smallest amount of scarlet fever antitoxin which will neutralize 50 skin-test doses of scarlet fever streptococcus toxin (when both are mixed together, allowed to stand for several minutes, and then injected intradermally). The further regulations of the National Institute of Health are that " . . No antitoxin for scarlet fever shall be distributed unless proper tests have shown that 1 cc. of the final product will neutralize and hold in neutraliza-

tion for 48 hours a minimum of 10,000 skin-test doses of the control toxin"; in other words, at least 200 units of antitoxin (as the measurement of potency is expressed now in terms of units) must be present in each cc. The U. S. Pharmacopoeia requirement is not less than 400 units per cc. These intracutaneous injections in a series of toxin-susceptible children for testing the potency of scarlet fever antitoxin are necessary as the toxin is not toxic for laboratory animals, and methods as used for standardization of diphtheria and tetanus antitoxins are not practical. Manufacturers in the United States of Scarlet Fever Streptococcus Toxin and Antitoxin, and other Scarlet Fever Streptococcus preparations are under license from the Scarlet Fever Committee, Inc. (which administers the patents issued to Drs. G. F. and G. H. Dick). The latter independently standardizes and tests all scarlet fever streptococcus preparations before release is granted for distribution. These manufactured products must also meet the following requirements of the National Institute of Health.

MINIMUM STANDARD REQUIREMENTS FOR SCARLET FEVER STREPTOCOCCUS ANTITOXIN

● The following requirements were issued by the National Institute of Health as of January 1, 1938:

"1. Only those sera shall be labelled Scarlet Fever Streptococcus Antitoxin which result from immunization of an animal with a strain of hemolytic streptococcus, or its metabolic products, whose erythrogenic toxin is neutralized by human convalescent scarlet fever serum.

2. The label for each final container shall state the number of units of antitoxin contained therein as determined by the approved tests, using the National Institute of Health standard toxin and antitoxin for control purposes.

3. No scarlet fever streptococcus antitoxin shall be distributed unless the approved tests have shown that 1 cc. of finished serum contains 400 units of antitoxin, or more.

4. A protocol showing the neutralizing value of each lot of antitoxin intended for the market shall be submitted to the National Institute of Health and the lot shall not be distributed until passed by the National Institute of Health as satisfactory. The protocol shall show the results of satisfactory titrations on at least three suitable test individuals who are susceptible to one skin test dose of scarlet fever streptococcus toxin. These tests shall be controlled by similar tests made on the same test subject at the same time and in corresponding locations using the control antitoxin which is distributed by the National Institute of Health. The results of all tests which are recorded on the protocol for one test subject must represent injections made on the same date.

5. The statement 'No U. S. standard of potency' need not appear on the label."

The following memorandum was issued on January 1, 1937, by the National Institute of Health:

"A Memorandum outlining the basic requirements for determining the neutralizing properties of Scarlet Fever Streptococcus Antitoxin.

1. Control antitoxin and control toxin are distributed by the National Institute of Health. It is requested that the control antitoxin be conservatively used so as to avoid unnecessary waste.

2. The final volume of each injection made for test purposes shall be 0.1 cc.

3. All mixtures of toxin and antitoxin must contain at least 5 skin test doses of toxin in the amount injected and in any event in a given test the number of skin test doses used must be the same for all toxin-antitoxin mixtures.

4. Each reaction resulting from an injection shall be measured in millimeters in its greatest diameter and at right angles to that diameter. Both measurements shall be recorded and the reaction shall be considered negative only if both measurements are less than 10 millimeters or if the product of the two measurements is less than 100.

5. A reading of the reactions shall be made 20-24 hours after the injections and again 40-48 hours after the injections and the determination of neutralization shall be based on the combined readings.

6. A test subject shall not be considered satisfactory for test purposes provided:

a. The reaction to one skin test dose of a control toxin measures less than that required for a positive reaction.

b. The reaction to either the control or test serum or both measures more than 4 millimeters in any diameter.

c. The reactions to all of the injected mixtures of standard antitoxin and standard toxin are positive.

d. There is a negative reaction to the mixture containing the least amount of control antitoxin.

7. A protocol submitted in accordance with paragraph 4 of the minimum requirements for Scarlet Fever Streptococcus Antitoxin dated January 1, 1937, (given above) shall show the following:

a. The reaction produced to the injection of one skin test dose of the control toxin.

b. The reaction or the absence of reaction following the injection of one or more mixtures of the new antitoxin with standard toxin.

c. The reaction and failure of reaction following the injection of at least two mixtures of control antitoxin and control toxin. The mixture containing the least amount of control antitoxin must give a positive reaction.
d. Reaction or failure of reaction following the injection of a quantity of antitoxin alone equal to the largest amount contained in any of the toxin-antitoxin mixtures injected. Such an injection shall be made both with the control antitoxin and the antitoxin under test. However, such control tests need not be made if the test individual is not liable to horse serum sensitivity.

8. The series of mixtures of control antitoxin and control toxin shall show a progressively increasing amount of antitoxin but in no instance shall the increase from one mixture to the next be more than 50%.

9. Each cubic centimeter of the glycerinated control antitoxin as distributed contains 40 units of antitoxin.

10. Calculation of the potency of the antitoxin under test is made from the protocol by comparing the end-point obtained with the new antitoxin with the end-point of the standard mixture on the same test subjeect. The calculation may be made for each individual test subject.

11. It is requested that protocols of tests made to determine potency be submitted in the form of the following protocol."

For Protocol—see page facing.

PROCEDURES FOR STANDARDIZATION

● For complete details for methods of standardizing scarlet fever antitoxins and toxin, see the method of Dick and Dick (*J. A. M. A.*, *84*, 803, 1477 (1925)), the method of Veldee (*U. S. Pub. Health Repts.*, *47*, 1043, (1932)), the rabbit intradermal methods (*J. Immunol.*, *28*, 33 (1935)), and the flocculation method (*J. Immunol.*, *32*, 321 (1937)).

DOSAGE AND USE

● In moderate cases of Scarlet Fever, 5,000 to 10,000 units of antitoxin are given intramuscularly as a therapeutic agent. In severe cases, 10,000 to 20,000 units are given intravenously, the dose generally being diluted with normal saline solution to 100 cc. Administration as early as possible in the disease course is advocated. Additional doses are given if the temperature does not fall within twelve hours to nearly normal. Numerous favorable reports have appeared regarding the use and value of Scarlet Fever Streptococcus Antitoxin as a specific curative agent. Its use results in a favorable effect on complications. There is usually a lessened severity and duration as well as incidence (Brit. Med. J., *1*, 351 (1939)).

For detection of serum-sensitive individuals and methods of desensitization, see under Diphtheria Antitoxin.

Scarlet Fever Antitoxin is also suggested as an emergency measure, for the temporary (passive) immunization of contacts who are Dick positive in doses of from 2,000 to 4,000 units. The resulting immunity, like that following the use of other serums (passive immunity) will endure for a period of time lasting not more than two or three weeks. Park, on the other hand, states that he seldom uses antitoxin as a preventive in scarlet fever contacts. He employs rather active immunization as the response he claims is very quick.

If prophylaxis to scarlet fever or treatment in mild cases is needed, some workers prefer to use an adequate dose of convalescent serum. They claim that in this country at this time scarlet fever is only a mild disease and children should not be unduly subjected to the risk of serum disease and future sensitivity to horse serum.

PROTOCOL

Name of Manufacturer

Potency Test on Scarlet Fever Streptococcus Antitoxin

Name of Operator ....

Date of Test	Case, Name or Number	Hour of Reading	Antitoxin Lot No.					National Institute of Health Antitoxin Lot					National Institute of Health Toxin 1 S.T.D.	Number of S.T.D. Neutralized by each c.c. of Manufacturer's Antitoxin
			..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.	..... c.c.		
				5 S.T.D. Toxin	5 S.T.D. Toxin	5 S.T.D. Toxin	5 S.T.D. Toxin		5 S.T.D. Toxin	5 S.T.D. Toxin	5 S.T.D. Toxin	5 S.T.D. Toxin		
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Note.—Measurements of two diameters of each reaction must be recorded in millimeters. Give the fraction of a cubic centimeter of antitoxin which is injected in each instance.

SCHULTZ-CHARLTON PHENOMENON

Blanching Test for Diagnosis of Scarlet Fever

The local blanching of the rash of scarlet fever by the intracutaneous injection of serum from a patient convalescent from scarlet fever, (by injection of 0.1 cc. of the latter into a region of the skin where the erythema is present), is known as the Schultz-Charlton phenomenon (*Ztschr. f. Kinderh.* *17*, 328 (1918)) and is regarded as a specific toxin-antitoxin reaction. This has a practical application and may be of decisive value as an aid in the diagnosis of scarlet fever especially in its early stages. It thus becomes possible to differentiate the rash of suspected cases of the latter from that of other eruptive fevers or rashes manifested by drug or food idiosyncrasies. It has been proven that this Schultz-Charlton reaction, or blanching phenomenon, can be induced by using Scarlet Fever Streptococcus Antitoxin instead of convalescent serum. In the test, 0.1 cc. or 0.2 cc. of the former is injected intradermally into the reddened skin of the suspected scarlet fever case and after twelve to twenty-four hours, in a positive reaction, there will be apparent a permanent blanching several centimeters in diameter around the site of the injection (best observed at some distance from the patient). This will persist for the duration of the eruption. There will be no blanching or whitening if the rash is not due to scarlet fever, though the absence of a positive reaction does not negative the diagnosis. For more details concerning this blanching phenomenon, see *Lancet, 2,* 1390 (1923) and *Arch. Dermat. u. Syph., 164,* 761 (1932).

VI

OTHER ANTITOXINS

GAS GANGRENE ANTITOXIN

Perfringens Antitoxin; Antitoxinum Welchicum (B.P.); B. Welchii Antitoxin; Anti-Gas Gangrene Serum

THIS is an antitoxic serum or a preparation of the latter (refined and concentrated serum consisting of the globulins to which are attached the antitoxic substances) which has the specific power of neutralizing the soluble toxin produced by *C. welchii.* The horses or suitable animals are immunized with gradual increasing doses of a sterile filtrate from a liquid culture of *C. welchii* (containing the toxin) or with an anaculture or with a toxoid (*Comp. rend. soc. biol., 92,* 1484 (1925)). After the desired degree of potency is obtained (as determined by potency tests applied to trial bleedings), the horses are bled, the blood allowed to clot, and the serum is collected. The antitoxic serum may be used in this liquid (unconcentrated) form, or it may be marketed as a refined and concentrated preparation by separating the globulins by fractional precipitation in a manner similar to that employed for preparing diphtheria and other antitoxins. The process of refinement by a controlled method of selective digestion with pepsin is practiced by one American manufacturer. The finished preparation is tested and standardized according to the method established and described by the National Institute of Health.

A unit of perfringens antitoxin is that amount necessary to neutralize 1000 M. L. D.'s of *C. welchii* toxin, an M. L. D. of the latter being the smallest amount of toxin which will kill a 300 gram pigeon when injected intramuscularly. For recent methods of standardization, see below.

GAS GANGRENE ANTITOXIN (COMBINED OR MIXED)

● The Combined or Mixed Gas Gangrene Antitoxin or Polyanaerobic Antitoxin or Anaerobic Antitoxin is the preparation most frequently marketed and used in the prophylaxis and treatment of gas gangrene. This antitoxic serum is prepared from the toxins of *C. tetani, C. welchii* (*C. perfringens*), and *C. oedematis maligni* (*Vibrion septique*). At times the soluble toxins of only two of the organisms mentioned may be used in the preparation of the marketable product or immunization may be against some of the other anaerobes concerned in gas gangrene, (*C. oedematiens; C. sordelli* (*C. oedematoides*); *C. histolyticum,* etc.). The different toxins are prepared individually. Horses or suitable animals are immunized with injections of but one toxin and the different antitoxins after preparation are mixed together. In some instances the same animal is immunized against several toxins simultaneously.

The products are supplied in refined and concentrated (Globulin) form and in unrefined form (liquid serum). The labels of the marketable Gas Gangrene Antitoxin (Combined or Mixed) indicate the different specific antitoxic substances and the amounts of each present. There is as yet no agreement as to the employment of an International standard or a uniform unit of measurement for determining the potency of these products, though many are participating in a project to promulgate an International standard (*U. S. Pub. Health Reports* Reprint 1735). In the United States, the unit values of the individual constituents in these polyvalent antitoxic serums are given separately and are in accordance with provisional standards established by the National Institute of Health. The American provisional unit is stronger than the proposed British and other foreign provisional units of Vibrion Septique Antitoxins.

TESTING AND STANDARDIZING GAS GANGRENE ANTITOXINS

● The increased use of gas gangrene antitoxins has stimulated studies in an attempt to establish more desirable standards and units of measurement for determining the potency of these products. A standard antitoxin and a standard toxin have been prepared for use in determining the potency of the following: Vibrion Septique Antitoxins (*U. S. Publ. Health Rep., 49,* 251-262 (1934)); Perfringens Antitoxin (*U. S. Publ. Health Rep., 49,* 525-529 (1934)); Oedematiens Antitoxin (*U. S. Publ. Health Rep., 51,* 266-275 (1936)); and Histolyticus Antitoxin (*U. S. Publ. Health Rep., 51,* 1263-1272 (1936)). The standards for use in this country have been prepared by the National Institute of Health and the respective potencies have been measured also in terms of International Units, the preparation prepared in the laboratory of the State Serum Institute at Copenhagen, (*Quart. Bull. Health Organiz., League of Nations,* Special Number, Jan. 1935). The tests are now carried out by either inoculating mice (intravenously) or by the intracutaneous inoculation of guinea pigs. For details of the methods of testing, the references quoted should be examined. The data presented is subject to change and manufacturers of these products usually withhold distribution pending the receipt of a release from the National Institute of Health. Releases are made on the basis of protocols furnished by the manufacturer and of tests made by the laboratory workers at the National Institute of Health.

The International Standard Unit as accepted by the Health Organization of the League of Nations for the following are given:

Cl. Welchii Antitoxin (*Health Org., Leag. of Nat., Rep. C. H., 1,* 1056 (1931)) unit is the specific antitoxic activity contained in 0.322 mgm. of a dry stable antitoxin prepared by the National Institute of Health in Washington.

Cl. Oedematis Maligni Antitoxin (*Quart. Bull., Health Org., Leag. of Nat.,* Spec. No., Jan. 1935, p. 13) unit is the specific antitoxic activity contained in 0.2377 mgm. of the dry standard preparation prepared at the National Institute for Medical Research, London, from material supplied by Dr. Weinberg of the Pasteur Institute.

Cl. Oedematiens Antitoxin. (*Quart. Bull., Health Org., Leag. of Nat.,* Spec. No., Jan. 1935, p. 42) unit is the specific antitoxic activity contained in 0.2681 mgm. of the dry standard antitoxin prepared at the Statens Serum Institute, Copenhagen.

Cl. Histolyticum Antitoxin (*Quart. Bull., Health Org., Leag. of Nat.*, Spec. No., Nov. 1936, 659-690) unit is the specific antitoxic activity contained in 0.3575 mgm. of the dry standard antitoxin as established by the Statens Serum Institute, Copenhagen.

A flocculative reaction between the toxin of *Cl. welchii* (Type A) and its antitoxin has recently been reported. A close correlation is shown to the mouse protection values of perfringens antitoxin. As an *in vitro* test this may replace or at least as a preliminary test may reduce the cost of the present assay methods of perfringens antitoxins which depend upon the use of living animals (*Proc. Soc. Exptl. Med. Biol., 40,* 620 (1939)).

USE

● Gas Gangrene Antitoxin and the various Gas Gangrene Antitoxins (Mixed) (so-called Polyanaerobic Antitoxin) are being used as prophylactic agents in those conditions in which the organisms commonly found causing gas gangrene (acute traumatic emphysematous gangrene) may occur, as in lacerated cartridge, gunshot and operative wounds, compound fractures, and before operation in cases of gangrenous appendicitis, peritonitis, intestinal obstruction and in puerperal infections, and in all cases involving severe trauma, crushed bone and deep perforations.

For prophylactic use, one injection is given subcutaneously or intramuscularly in the region of the wound. Two or three injections at weekly intervals may be necessary if the wound is slow in healing. Each injection consists of 2500 to 4000 units of gas gangrene antitoxin or from 3000 to 8000 total units of the gas gangrene antitoxin (mixed).

For therapeutic use, initial doses of from 4000 to 10,000 units intramuscularly and 4000 units intravenously are given followed by 4000 to 8000 units intramuscularly at daily intervals as indicated.

Coller (*Military Surgeon, 81,* 27 (1937)) advocates the intravenous administration of a polyvalent serum well diluted in normal saline or glucose solution as the therapeutic method of choice. Malone (*J. Tenn. M. A., 30,* 402 (1937)) found that the marketed polyvalent gas gangrene antisera were not efficient.

A definite clinical evaluation of these gas gangrene antitoxins is impossible at present. It has been suggested that serums against gas gangrene infections should be antibacterial as well as antitoxic. More extensive use of these antitoxic sera and future investigations alone will make possible a proper evaluation of serum therapy in gas gangrene.

In a recent report on the morbidity and mortality of gas gangrene in New York State (exclusive of New York City) the findings reveal that this type of infection probably occurs as frequently as tetanus. It has been suggested that free gas gangrene antitoxin should be distributed inasmuch as the favorable effects of its use are indicated by the literature (*N. Y. State J. Med., 38,* 1022 (1938)).

Where there is any question of doubt, polyvalent antitoxins are to be used. The inclusion of tetanus antitoxin in the injected material is to be given due consideration. A monovalent serum is employed in those instances where a bacteriological examination revealed a monobacterial infection. If a mixed antitoxin is used as the initial injection, the kind of antitoxin to be used in later injections can be changed to correspond to the bacterial flora present. Speaking generally, it appears that the use of anaerobic antitoxin reduces the mortality from infections caused by these anaerobes (*Arch. f. klin. chir., 179,* 312 (1934); *Indust. Med., 5,* 234 (1936); *Ann. Surg., 105,* 257 (1937)).

STAPHYLOCOCCUS ANTITOXIN

CULTURES and filtrates of liquid cultures of certain strains of *Staphylococcus aureus* and *albus* are known to be toxic. In fact five different toxic effects have been described. The problem which presented itself was whether these filtrates contain either five distinct toxins or whether there is present only one antigenic toxin with at least five distinct actions. The evidence which has accumulated has resulted in regarding the toxic filtrate as containing the following distinct and separate exotoxins: (1) Hemolysin; (2) Leukocidin; (3) Necrotizing (or dermonecrotic) toxin; (4) Lethal toxin; and (5) Gastro-enteric toxin concerned in food-poisoning.

Dolman (*Canad. Med. Assoc. J.*, *30*, 601; *31*, 1 (1934)) in his investigations detected in the filtrates of staphylococci grown under suitable conditions the presence of a hemolysin, a leukocidin, a necrotoxin, a plasma-coagulating agent, and a substance producing rapid death when injected intravenously into laboratory animals. The erythrogenic and lethal properties were demonstrated by other workers.

ANTITOXIN

● Extensive investigations have been carried out recently on the preparation, effects and value of staphylococcus toxin, toxoid and antitoxin, the latter being advocated in cases of staphylococcus toxemia or septicemia (*J. Missouri M. A.*, *33*, 453 (1936)); (*J. A. M. A.*, *100*, 1007; Apr. 20, 1421 (1934)); (*Compt. rend. soc. biol.*, *117*, 717 (1934); *118*, 212, 1062 (1935); *122*, 1002, 1004 (1936); *124*, 315, 898 (1937); *125*, 128 (1937)); (*Lancet*, *226*, 1054 (1934); Feb. 9, 303, 306 (1935)). The marketable staphylococcus antitoxins are prepared from the refined and concentrated globulins obtained from the serum of horses injected with staphylococcus toxin or toxoid.

A concentrated staphylococcus antitoxin containing antibacterial antibodies which is at times designated as *Staphylococcus Ambo-Antibody Solution* is also available. The latter is prepared by injecting horses with a so-called *staphylococcus ambotoxoid* (*Proc. Soc. Exptl. Biol. and Med.*, *34*, 502 (1936); *Am. J. Publ. Health*, *26*, 1001 (1936)). The ambotoxoid known also as *staphylococcus bacteriophage toxoid* is a staphylococcus antigen prepared by reinoculating staphylococcus toxin with pooled cultures of suitable staphylococci (aureus and albus) and also with an adapted staphylococcus bacteriophage (*J. Immunol.*, *29*, 209 (1936)). After clearing the culture a preservative is added, usually formalin and merthiolate. After incubation until detoxification, the finished product is tested for sterility, potency and toxicity. The antibodies produced by the administration of the ambotoxoid are antitoxic as well as antibacterial. In the preparation of the antitoxin for marketing, the procedure is almost identical as is practiced for other antitoxins.

U. S. Standard Staphylococcus Antitoxin

A United States standard staphylococcus antitoxin has been prepared and dried by the freezing and vacuum method. The unit of antitoxic potency of the United States standard staphylococcus antitoxin is based on and is equal to the unit of the International Standard Staphylococcus Antitoxin. One unit is contained in 0.0009502 grams of the United States standard staphylococcus antitoxin. For distribution purposes the dried standard staphylococcus antitoxin is dissolved in a 60% glycerol solution containing twenty staphylococcus antitoxin units per cubic centimeter of solution.

Methods of Testing

Three methods of testing the potencies of staphylococcus toxin-antitoxin mixtures are in use at present: (a) hemolytic; (b) dermonecrotic; (c) lethal. The following procedures are used by the National Institute of Health.

The Hemolytic Test

A normal rabbit is bled from the heart and the blood shaken with sterile glass fragments or beads. Five c.c. amounts are accurately measured into small, graduated centrifuge tubes. Sterile physiological saline solution is added and centrifuged at a fairly low speed so as to avoid mechanically injuring the erythrocytes. The cells are thus washed three times. After removing the final supernatant, the cells are made up with sterile physiological saline solution again to exactly 5 c.c. volume. To one c.c. of this blood is added 99 c.c. of a sterile physiological saline solution. One c.c. of the 1 to 100 dilution is used as the indicator for the hemolytic test. One unit of antitoxin is used in each tube to titrate against varying toxin dilutions. Utilizing 1/10 of a unit or 2/10 of a unit did not give results similar to one whole unit. The toxin dilutions are placed in small test tubes (½ inch by 3 inches) first, then the antitoxin is added and lastly the cells suspension. The total volumes are the same in all tubes. A self measuring syringe is used here for these purposes and for mixing the contents of the tubes. The tubes are incubated at 37° C for 1 hour, then placed in refrigerator (10° C) and readings made the next morning after all interaction has ceased and the red blood cells have settled. Six degrees of hemolysis are recorded: 4 plus is complete hemolysis; 3 plus hemolysis has as red a supernatant as the 4 plus but there may be seen a trace of settled, intact red cells; 2 plus hemolysis has definite color in the liquid due to dissolved hemoglobin, but half the original cells are not destroyed—they are still intact in the bottom; 1 plus hemolysis shows most of the cells in the bottom but the whole fluid has a trace of red; in plus-minus hemolysis practically all the cells are in the bottom and a water clear supernatant but just above the settled cells there is half an inch or so of red fluid. No hemolysis means all the intact red blood cells settled in the bottom and the supernatant water clear. Two plus hemolysis is considered the end point, and if four plus occurs at toxin dilution (say) 1/440 gram and 0 hemolysis at 1/460 gram dried toxin per c.c. of the dilution—the end point is considered to be about 1/450 gram.

The Dermonecrotic Test

White rabbits or white guinea pigs may be used. The former are more sensitive, the latter are more realible in that the results are more constant and uniform. The latter (guinea pigs) are used for official testing. Rabbits are bled from the ears and the serums tested by the hemolytic method to insure that the rabbit has a low degree of natural immunity (less than 1/10 unit per c.c.) to staphylococci. Guinea pigs serums are not tested preliminary to the test. The animals' backs are closely clipped or shaved and marked off into squares. Into each area is injected, intradermally, two tenths of a c.c. of a toxin-antitoxin mixture. The mixtures are not incubated before injection. Necrosis, half a centimeter in diameter or larger is considered to indicate an excess of toxin over antitoxin. Midway between that point and simple erythema or no reaction, at the end of four days, is considered to be the neutralization point. It is not often practical to use one whole unit of antitoxin in this test so one half, or other fraction (but not less than 1/10) of a unit, is used as the constant factor.

The Lethal Test

For the lethal test rabbits may be used but white mice are as satisfactory, cost less and many more animals may be used on a given test. The mice used here weigh from 17 to 22 grams each. Several mice—preferably ten—are injected intraperitoneally with 0.5 cc. volumes of each of the toxin-antitoxin mixtures. At the end point, half the mice die in three days.

Standardization

In the assay of staphyloccoccus toxins and staphylococcus antitoxins of unknown potencies, the standard antitoxin is the starting point—the fixed factor to which the unknowns are compared.

The test dose of a staphylococcus toxin is considered to be that amount of toxin that is just neutralized by one unit of the standard staphylococcus antitoxin. Tested by the hemolytic method, the test dose is designated by the symbol Lh, tested by the dermonecrotic reaction test, by the symbol LR, and tested by the lethal test, by the symbol L_{+}. These test doses of toxins are determined by mixing varying dilutions of toxin with a constant amount (usually one unit) of antitoxin and testing by the methods described previously.

The test dose of a toxin having been determined, it may be used as the fixed factor against which varying dilutions of unknown antitoxic serums may be tested. That dilution of antitoxin, in the test, which just neutralizes one test dose of toxin, contains one unit of antitoxin. Knowing the test amounts and the dilutions, the number of staphylococcus antitoxin units per c.c. of the original unknown serum may be computed. The protocols are as issued by the National Institute of Health.

The protocols are shown on succeeding pages.

● Levine (*J. Path. and Bact.*, *48*, 291 (1939)) reveals that titration of staphylococcal antitoxin for anti-hemolytic, anti-necrotic and anti-lethal activity gives identical results. Evidence is presented revealing that the lytic, necrotic and lethal properties of the toxin are due to the same toxic substance.

INTERNATIONAL STANDARDIZATION OF STAPHYLOCOCCUS ANTITOXIN

● In the Quarterly Bulletin of the Health Organization of the League of Nations, January 1935, are to be found the methods for the standardization of staphylococcus toxin and antitoxin and proposed international units.

Test to determine the hemolytic test dose (Lh) of an unknown staphylococcus toxin.

Unknown Staphylococcus Toxin (called "A") cc.	0.526	0.55	0.59	0.602	0.625	0.66	cc. 0.713
U. S. Standard Staphylococcus Antitoxin (units)	one unit	one unit	one unit	one unit	one unit	one unit	one unit
Total volumes same in all tubes.							
Hemolysis	0	1+	1+	2+	3+	4+	4+

0.0602 c.c. taken as the hemolytic (Lh) test dose of toxin "A".

Test to determine the antitoxic potency of an unknown serum utilizing the hemolytic test dose of toxin "A".

Unknown Staphylococcus cc. antitoxic serum (called "I")	1/540	1/560	1/580	1/600	1/620	1/640	1/660
Staphylococcus toxin "A"	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose
Total volumes same in all tubes.							
Hemolysis	0	+	1+	2+	3+	4+	4+

1/600 c.c. of staphylococcus antitoxin serum "I" taken as the amount containing one unit of antitoxin. Staphylococcus antitoxin "I" contains 600 units per c.c.

CONTROL

Staphylococcus toxin "A"	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose
U. S. Standard staphylococcus antitoxin (units)	0.85	0.90	0.95	1.0	1.05	1.10	1.15
Total volumes same in all tubes.							
	4+	3+	2+	2+	2+	1+	0

PROTOCOL A

The proposed *International Unit* of antitoxin is that amount which will neutralize 200 M. H. D. of toxin. The *Minimum Hemolytic Dose* of the toxin is that amount of toxin which will hemolyze 1 cc. of a 2 percent suspension of rabbit red cells in one hour incubation at 37° C.

STRENGTH

● The marketed staphylococcus antitoxins should contain not less than 250 antitoxin units per cc. They are usually supplied in sterile containers holding 10,000 units.

USE AND DOSAGE

● The average minimum dosage is 10,000 units given intramuscularly or intravenously for mild staphylococcic infections. For severe conditions and

Test to determine the dermonecrotic test dose (LR) of an unknown staphylococcus toxin

2/10 c.c. mixtures injected intradermally into each area of clipped backs of 5 white guinea pigs weighing 350 to 400 grams.

Unknown staphylococcus toxin (called "A") c.c.	0.0425 cc.	0.04 cc.	0.0375 cc.	0.035 cc.	0.0325 cc.
U. S. standard staphylococcus antitoxin (units)	5/10 unit	5/10 unit	5/10 unit	5/10 unit	5/10 unit
Total volumes same in all mixtures.					
Dermonecrosis	5/5	5/5	3/5	1/5	0/5

5/10 dermonecrotic test dose (LR/2) considered to be 0.0375 cc. toxin "A".

Test to determine the antitoxic potency of an unknown serum, utilizing the dermonecrotic test dose (LR/2) of toxin "A".

Unknown staphylococcus c.c. antitoxic serum (called "I")	1/1400 cc	1/1300 cc	1/1200 cc	1/1100 cc	1/1000 cc
Staphylococcus toxin "A" (test dose)	5/10 test dose	5/10 test dose	5/10 test dose	5/10 test dose	5/10 test dose
Total volumes same in all mixtures.					
Dermonecrosis	5/5	5/5	2/5	1/5	0/5

1/1200 c.c. of staphylococcus antitoxic serum "I" considered as the amount neutralizing 5/10 of a test dose of toxin. 10/10 or one test dose of toxin, presumably, would be neutralized by 1/600 c.c. serum. Staphylococcus antitoxic serum "I" considered to contain 600 units per c.c.

CONTROL

Staphylococcus toxin "A" (test dose)	5/10 test dose	5/10 test dose	5/10 test dose	5/10 test dose	5/10 test dose
U. S. Standard staph. antitoxin (1 cc = 10 units)	0.07 cc	0.06 cc	0.05 cc	0.04 cc	0.03 cc
Total volumes same in all mixtures.					
Dermonecrosis	0/5	0/5	2/5	5/5	5/5

The preceding test was performed on the left sides of five white guinea pigs and the control test on the right sides of the same guinea pigs.

PROTOCOL B

when complicated by staphylococcemia the initial dosage shall be not less than 20,000 units. Injections are to be given daily or at longer intervals depending upon clinical improvement. As when using other serums, administration should be given as early as possible. Intravenous therapy is indicated in severe infections, but testing for sensitiveness to horse serum should be practiced. In infections involving the central nervous system, intrathecal or intraventricular injections may be tried in addition to intramuscular or intravenous medication, though some workers do not advocate intrathecal therapy at any time.

VALUE

● Reports by some workers indicate that staphylococcus antitoxin is of some value in the treatment of acute generalized staphylococcus infections as well as in obstinate or spreading localized staphylococcus infections where the etiological factor was found and confirmed by bacteriological

Test to determine the lethal test dose (L_+) of an unknown staphylococcus toxin.
0.5 cc. toxin-antitoxin mixtures injected intraperitoneally into white mice (17 to 22 grams each)

Unknown staphylococcus toxin (called "A")—c.c.	0.11 cc	0.1	0.09	0.08	0.07	0.06	0.05 cc
U. S. Standard Staphylococcus antitoxin (unit)	one unit	one unit	one unit	one unit	one unit	one unit	one unit
Total volumes same in all mixtures.							
Dead in 3 days	10/10	10/10	9/10	4/10	0/10	0/10	0/10

One lethal dose (L_+) considered to be contained in 0.08 cc. of toxin "A".

Test to determine the antitoxic potency of an unknown serum, utilizing the lethal test dose (L_+) of toxin "A".

Unknown staphylococcus antitoxic serum (called "I") c.c.	1/900	1/800	1/700	cc. 1/600	1/500	1/400	1/300
Staphylococcus toxin "A" (test dose)	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose
Total volumes same in all mixtures.							
Dead in 3 days	10/10	10/10	10/10	6/10	1/10	0/10	0/10

1/600 c.c. of the unknown serum neutralized one test dose of toxin.
Staphylococcus antitoxin "I" considered to contain 600 units per c.c.

CONTROL

Staphylococcus toxin "A" (test dose)	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose	one test dose
U. S. Standard staphylococcus antitoxin (1 cc. = 10 units)	cc. 0.13	cc. 0.12	cc. 0.11	cc. 0.1	cc. 0.09	cc. 0.08	cc. 0.07
Total volumes same in all mixtures							
Dead in 3 days	0/10	0/10	0/10	6/10	9/10	10/10	10/10

PROTOCOL C

examination. Dolman (*Canad. M. A. J., 30,* 601; *31,* 1 (1934)) reported on a series of 104 staphylococcic infections affecting almost all body tissues and recommends the use of antitoxin. Nelis and Mechelen (*Roy. Hyg., 58,* 729 (1936)) suggest antitoxin therapy for furunculosis in miners. In experimental staphylococcic infections in rabbits, Rigdon (*J. Lab. Clin. Med., 23,* 159 (1937)) found that the antitoxin succeeded in prolonging the life of the animals beyond that of the controls. Parker (*J. Missouri M. A., 35,* 30 (1938)) reports on the life-saving property of staphylococcus antitoxin and suggests that specific treatment of septicemia with this preparation is indicated. On the other hand Jamieson and Powell (*Am. J. Hyg., 19,* 246 (1934)) and other workers report that the antitoxin did not produce uniformly good results. Inasmuch as staphylococcus antitoxin does not appear to possess a demonstrable bactericidal effect, the use of this preparation may be limited and it does not appear that it ever will be widespread unless a more potent therapeutic antitoxin or serum will be made available.

BOTULINUS ANTITOXIN

Anti-botulinus Serum; Botulism Antitoxin; Botulinus Antitoxin Serum; Serum Anti-botulinum

Clostridium botulinum may be divided into several types which though culturally very similar to each other are differentiable by the individual specificity of their toxins and therefore the corresponding antitoxins. The usual types found in this country are those designated by the nomenclature of types A, B, and C. Types A and B are associated with human botulism. Type C strains are apparently non-pathogenic for man *(J. Infect. Dis.,* 1921, VIII, 226). Types D and E, described recently, are associated with certain animal diseases.

In North America, type A is the most frequent cause of botulism, whereas in Europe type B is found most frequently. Only one outbreak of botulism has been recorded from Great Britain (in Scotland during 1922 (*Brit Med. J.* 1923, I, 279)). 146 outbreaks with a total of approximately 500 cases and a 65% fatality rate have been reported in the United States and Canada from 1909 to 1926 (Hewlett, R.T.—"*A System of Bacteriology*", 1929, London, *H. M. Stationery Office,* III, 374). Botulinus toxin is extraordinarily potent and remarkable in its ability to withstand the action of heat and body juices. Its action in the body after an incubation period, reveals the following symptoms: dyspnea, blurred or double vision, giddiness, convulsions, paralysis of deglutition and respiration, occulomotor paralysis, constipation, and death is due to respiratory paralysis *(Monogr. Rockefeller Inst. M. Research,* No. 8, 1918). The toxin is preformed and intoxication follows the ingestion of food containing the toxin.

PREPARATION

● Antitoxin has been prepared for each of the specific types and it is important to note that antitoxin produced against one series of strains (type A) has no appreciable effect upon the toxins of the strains of the other types (types B). The two types of antitoxin which are most commonly employed are prepared by immunizing animals (horses or cattle) against the toxin of each type (*C. botulinum type A and B*) separately. The procedures for the production of the toxin and antitoxin correspond closely to those given in the technique for producing tetanus antitoxin, except that the whole serum (and not concentrated globulins) is the marketed product. The preservative consists of a mixture of equal parts of refined tricresol and ether so that the final volume is 0.8 percent of the combined preservative.

An M.F.D. or M.L.D. of botulinus toxin is the smallest amount of toxin which when injected subcutaneously will kill a 250-gram guinea-pig within ninety-six hours. Botulinus antitoxic serums are standardized by determining their neutralizing value when mixed with the homologous botulinus toxins previously standardized against the official standard botulinus antitoxic serum supplied by the National Institute of Health (*Am. J. Pub. Health,* 1921, XI, 352). The two antitoxins (types A and B) can be mixed (pooled) to yield a polyvalent antitoxin for use in botulism. Some workers prepare a polyvalent antitoxin by immunizing a single horse with toxin prepared from all types of *C. botulinum.* The former technique is advocated however as being more satisfactory.

USE

● As a therapeutic agent for human cases Dickson advises intravenous injection after the sensitivity to horse serum has been eliminated (*J. Exp.*

Med., 1923, XXXVIII, 327). Comparative large doses (50 cc. (10,000 units) daily) of Botulinus Antitoxin (bivalent serum or of the monotypical serum) types A and B are given (at the rate of not more than 1 cc. per minute) until recovery. The results of the use of Botulinus Antitoxin in man have been disappointing. However since no other effective treatment for botulism is known, the antitoxin should be given a trial. Success can only be expected from serum therapy if the antitoxic serum is administered in the early stages of the disease or preferably as soon as suspicious symptoms appear (Meyer, K.F., 1928, *"Handbuch der pathogenenen Mikro-org."*, *Band IV*, 1343). Individuals who have consumed food suspected of being infected are given prophylactic doses of 10 cc. subcutaneously (or at least 2,000 units) intramuscularly.

In terms of units, the usual prophylactic dose is at least 2,000 units of the bivalent antitoxin given subcutaneously. The therapeutic dose is at least 10,000 units of the bivalent antitoxin given intravenously and repeated as indicated. The most frequently used marketed product contains 2,500 units each of type A and type B botulinus antitoxin.

In Hygienic Laboratory Bulletin, No. 136 (1924), the following instructions for the use of the antitoxin are given as having been issued by the Ministry of Health of Great Britain:

"Botulinus Antitoxic Serum:—Instructions as to Use

"1. *Immediate administration.*—The only remedy at present known for botulism is botulinus antitoxin given by injection and even this is unlikely to save life if the disease has progressed to a late stage. It is therefore of the greatest importance *to give the antitoxin at the earliest possible moment*—i.e., as soon as the earliest symptoms of blurred or double vision, giddiness, ptosis, difficulty in speech, or swallowing suggest the diagnosis of botulism.

"2. *Method.*—The effectiveness of treatment by antitoxin for botulism is so greatly increased by intravenous injection that this method of administration should always be employed (otherwise the intramuscular method should be used). The risk of serious symptoms arising in persons highly sensitive to horse serum must be recognized, and when, in the judgment of the medical practitioner, such is likely to be great, he should administer a preliminary injection of a small quantity of the antitoxin (0.5 cc.—8 minims) *subcutaneously.* If this produces clear evidence that the patient is sensitive (that is, if a reaction appears in half an hour) then the antitoxin must be given with particular caution, the first intravenous injection consisting of 0.5 cc. of antitoxin being diluted with 50 cc. of saline, and subsequent injections being diluted less and less till the full dose has been administered.

"3. *Dose.*—Only large doses will save acute cases. The intravenous doses should be from 20 to 50 cc. of the antitoxin, previously warmed to body temperature and injected very slowly; this amount may be repeated daily if necessary.

"*NOTE.*—All other persons who have consumed the suspected food but have not yet presented symptoms should be given a prophylactic dose of antitoxin (i.e., 10 cc.) intramuscularly, to be followed by larger doses intravenously should symptoms appear."

DISSOCIATION OF TOXIN AND ANTITOXIN

It is important to note that the union between the toxin and antitoxin is not a destructive action but a binding one. The latter becomes closer

with time but in the earlier stages dissociation is possible by various means. Dilution, introduction into the stomach or the addition of acid may result in the dissociation of a neutralized toxin-antitoxin mixture with the result that the fraction so treated is more toxic than the mixture itself.

STANDARDIZING AND TESTING

● A dried toxin of each of the types of A and B was used originally as the basis for determining the standard unit. One hundred M. L. D.'s of the latter were used as a *test dose* preliminary to establishing the unit of antitoxin. Potent serums prepared from each of the types were admixed with glycerin in the proportion of one part of serum to two parts of glycerin. The smallest amount of glycerinated serum (antitoxin) of each type, which when mixed with the test dose of its corresponding toxin and injected into a 250-gram guinea pig causes the death of the guinea pig within 96 hours is said to contain one-tenth of a unit of antitoxin. In other words, a unit of standard antitoxin just fails to neutralize approximately 1,000 M. L. D.'s of the original dried toxin. The standard unit of antitoxin distributed by the National Institute of Health is contained in 0.4 cc. and prepared from tested antitoxic serum diluted with glycerin. The following gives a condensed satement of the definition of the unit and method of testing (*Hyg. Lab. Bull.*, No. 136):

"The unit of botulism antitoxin (type A) is a definite amount of the standard antitoxin; for the standard antitoxin in the concentration used at present and to be sent out to serum establishments which may be licensed for the production of botulism antitoxin, the unit is 0.4 cc. of the glycerinated solution. The antitoxin to be tested is compared with the standard unit by means of a test dose of toxin; this test dose of toxin is chosen so that when mixed with one-tenth of a unit of the standard antitoxin and injected subcutaneously into a 250-gram guinea pig, the guinea pig will die in about 96 hours. That amount of the unknown antitoxin which when similarly mixed with the test dose of toxin and injected is just sufficient to protect a 250-gram guinea pig contains one-tenth of a unit. The test dose of toxin will usually be found to contain from 50 to 200 minimal lethal doses. Before injection the doses of toxin and antitoxin are to be thoroughly mixed, and then kept at room temperature for one hour to allow combination to take place. The guinea pigs should be between 250 grams and 300 grams in weight, and the doses of toxin and antitoxin are to be increased proportionately to the weight for guinea pigs weighing over 250 grams. With every test of an unknown antitoxin, four control guinea pigs should receive the test dose of toxin mixed with the test dose (one-tenth unit) of standard antitoxin so that the two antitoxins may be accurately compared and the test dose of toxin increased if necessary in subsequent tests; liquid toxins have been found to decrease gradually in toxicity. In diluting the standard antitoxins, the method indicated on the label should be followed. The first dilution should be made with a capacity pipette, to be washed out in the diluting fluid, on account of the viscosity of the glycerinated serum.

The units of botulism antitoxins (types B and C) are similarly definite amounts of the standard types B and C antitoxins to be sent out to serum establishments which may be licensed for the production of these antitoxins, the unit of each being 0.4 cc. of the glycerinated solution. Antitoxins to be tested are compared with the standard units by means of test doses of types B and C toxins, using the same method as above described for type A."

ERYSIPELAS STREPTOCOCCUS ANTITOXIN

Erysipelas Antistreptococcus Serum; Erysipelas Streptococcus Antitoxin, Refined and Concentrated

IN 1883, Fehleisen *(Die Aetiologie des Erysipels, Berlin, 1883)* isolated a streptococcus from erysipelas lesions and reproduced the disease in man by these organisms from pure cultures. Being of the opinion that this streptococcus was a distinct species, he named it *Streptococcus erysipelatis*. Other workers have opposed this opinion and the exact status as to specificity of erysipelas streptococci is not definitely settled. Birkhaug in 1925 *(Johns Hopkins Bull.*, 1925, XXXVI, 248; XXXVII, 307) presented the beginning of the more recent evidence in favor of regarding streptococci from erysipelas lesions as a distinct group and species. He showed that these streptococci produce a soluble toxin which is specifically neutralizable by antitoxic serum obtained from animals immunized against this toxin. An antiserum produced in horses against the toxin of streptococci from cases of erysipelas has been used successfully in the treatment of this disease (*South Med. J.*, XXIII, 29, Jan. 1930), (*Am. J. Dis. Child.*, *39*, 529 (1930)). McCann (*J. A. M. A.*, 1928, *91*, 78) in a critical review expresses doubt as to the value of this serum.

The preparations marketed today under the title of Erysipelas Streptococcus Antitoxin or Erysipelas Streptococcus Serum are prepared from the blood of horses which have been immunized not only against the toxin but also against highly virulent cultures of streptococci isolated from erysipelas. The untreated immune serum or the latter refined and concentrated (and freed of most of the non-active serum constituents) is employed. In both instances the finished and marketable preparation contains not only the antitoxic but also the antibacterial immune bodies. The activity is determined and the preparation is standardized by testing its neutralizing power against a specific test toxin employing a technique similar to that used for testing Scarlet Fever Streptococcus Antitoxin. Another method yields findings in terms of units, a unit of Erysipelas Streptococcus Antitoxin in this technique being regarded as 1-10 of the amount of antitoxin required to protect 66 percent of the mice injected with an M.F.D. (or M.L.D.) of a virulent culture of *Streptococcus erysipelatis*. The relative value of these sera is still under investigation. Reports have appeared indicating that Erysipelas Streptococcus Antitoxin or Serum is of value in favorably affecting the course of erysipelas by lowering and reducing rapidly the temperature, decreasing leucocytosis, causing a disappearance of albumin in urine, and a rapid fading out of the lesions together with marked improvement in the symptoms of toxemia (*J. Pediatrics*, 1934, IV, 746). Best results are obtained if the antitoxin is used early (*J. A. M. A.*, May 8, 1926, p. 1411). There is no established dosage. The quantities recommended are 10 to 20 cc. injected intramuscularly or intravenously in mild cases or daily doses or even every twelve hours for the first four injections and then every twenty-four hours until a permanent improvement is noted or the condition is brought under control. Each cc. contains 50,000 protective units.

Wadsworth (*J. A. M. A.*, 1932, *99*, 204) reports on a monovalent serum prepared from Dochez's "N. Y. 5" strain of scarlet fever streptococcus and reveals its beneficial effect in 67% of 106 severe cases of erysipelas. The toxin from this scarlet fever strain of Dochez "N. Y. 5" together with the toxin produced by typical strains of streptococci isolated from erysipelas lesions are used for the immunization of horses and the prepa-

ration of a marketable *"Erysipelas Streptococcus Antitoxin, Globulin Modified"*. A process of digestion is employed which depends upon the treatment of the proteins in the immune horse blood with pepsin. Ninety percent of the coagulable proteins are digested by this process, a smaller portion is precipitated and the remainder, a pseudoglobulin fraction, is further purified by ultrafiltration and dialysis. The finished product is subjected to the usual rigid tests and is marketed in sterile syringes containing *"one basic dose"*. The latter is generally administered intramuscularly in early cases of moderate severity and repeated once daily if necessary until the erysipelatous blush disappears. Large doses at more frequent intervals are advocated in late and severely toxic cases.

See also *Meningococcus Antitoxin,* page 75.

VII

SERUM ANTIVENENOSUM

Snake Poison Antitoxin

Anti-venom Serums; Serum Antivenenosum; Antivenenes; Antivenins

THE poisonous snakes differ from the non-poisonous snakes mainly in the fact that many or few of the maxillary teeth assume the character of poison-fangs and they also possess two supralabial glands (corresponding to the parotid salivary glands of higher animals), one on each side of the head beyond the eye above the lip margin and which secrete the poison or venom. The fangs are larger than the ordinary teeth found in snakes, vary in size from one-quarter to one inch in length, and are grooved or perforated by a canal (as in a hypodermic needle) for the passage of the duct of the poison-gland. Venom does not flow freely from the fangs of poisonous snakes except during the act of striking. The degree of danger from a bite depends not only upon the species of poisonous snakes which inflicted the wound, but on the size and age of the snake, the length of the period of hibernation, fasting, repose and other environmental factors which influence the amount of poison or venom deposited and finally the place where the wound was made. The deathly effect of the venom is in proportion to the body weight of the one bitten, so that an amount which when injected into a large body or that of an adult may not prove fatal would, however, in the same dose, kill a child.

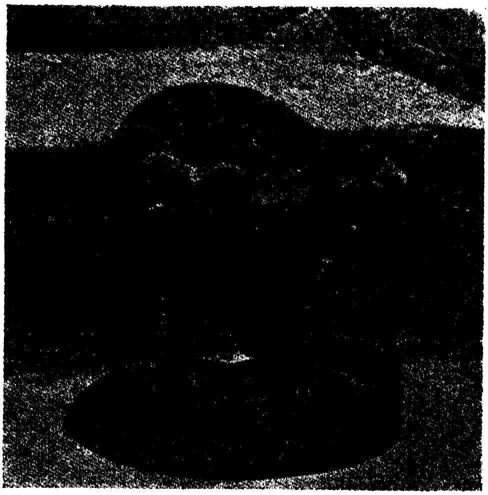
Excluding the Sea Snakes, which are merely Water Cobras (and all are venomous), there are about 250 other species of snakes which have a well developed poison apparatus. About one-third of this number may be disregarded, due to their rarity or small size. (For more details concerning snakes, see *Amer. J. Pharm.*, 1929, Vol. 101, No. 6 and 7). The poisonous snakes belong chiefly to the Viperine and the Colubrine families (*Viperidae*, in which are to be found the two important subfamilies, *Viperinae* and *Crotalinae*; and *Colubridae*, in which are to be found the subfamilies, *Hydrophinae* (or Sea Snakes) and *Elapinae*). The Elapine snakes include the Cobras (genus, *Naja*), which contains King Cobra or Hamadryas and many other species widely distributed over Asia and Africa, the Kraits (genus, *Bungarus*) found in India and adjacent areas, and the Coral snakes (genus, *Micrurus* or *Elaps*), found in southern United States, Mexico, Central America and tropical South America. The Death Adder (*Acanthophis antarcticus*), one of the important poisonous snakes found in Australia, is another dreaded Elapine snake. Among the Viperinae or true

vipers, observed mainly in the Old World are to be found the Common Viper (*Vipera berus*), the only poisonous snake inhabiting the British Isles; Russel's Viper (*V. russelli*) found in India and the East Indies; and the Puff Adder (*Bitis arietans*), widely distributed in Africa. Among the Pit Vipers or Crotalinae are found most of the poisonous snakes (all except the Coral snakes) observed in the United States as the several species of rattlesnakes (listed in two genera, *Sistrurus* and *Crotalus*), the Water Moccasin or Cottonmouth snake (*Agkistrodon piscivorus*) and the Copperhead snake (*Agkistrodon mokasen* or *A. contortrix*). The genus Lachesis or Bothrops also belongs to the Crotalinae or Pit Vipers. This genus contains many species found only in Central and South America and Southeastern Asia. Here we find the Fer-de-Lance (*Bothrops atrox* or *L. lanceolatus*) and the Bushmaster (*Bothrops mutus*).



ANTIVENIN OR SNAKE VENOM ANTITOXIN

● As in the manufacture of antitoxins specific for the soluble toxins or poisons produced by bacteria, snake venom antitoxin (antivenin) can be and is produced which is specific for the snake poison (or venom) employed in its production. *It has been found impossible or impractical to prepare an antivenin specific for the venom of all poisonous snakes.* Evidence has been presented however which reveals that there exists a high degree of generic specificity among the snake venoms. It is thus possible to prepare an antivenom serum or antivenin, which will be of practical value and which will be polyvalent for the reason that the venom of many species of poisonous snakes belonging to the same genus or to closely related genera is employed in its manufacture.



To the left, Agkistrodon piscivoris, "Cotton-mouth Moccasin." Above, Crotalus atrox, "Western Diamond Rattlesnake," (showing position for striking).

Snake venom antitoxins or anti-venom serums or antivenins are marketed which are either monovalent (of value for the specific venom used for its

production) or polyvalent (of value for the venoms obtained from several species of poisonous snakes belonging to the same family). *Calmette's anti-venene* for cobra venom (*Jour. Med. Res.*, 1909, XXI, 47) prepared today by the Pasteur Institute at Saigon is of value in the treatment of cobra envenomation, but valueless for the treatment of the venom of other poisonous snakes. In England, *Anti-Adder Serum* is marketed. It is prepared from and employed to neutralize the venom from the Common Viper (*Viperus berus*). Such a monovalent serum may prove satisfactory in the British Isles as this is the only poisonous snake found in that locality. At Kasauli in India, a bivalent serum is prepared against the venom of the cobra and that of the daboia. In Australia, an antivenin is issued possessing a high potency against the venom of the tiger snake (*Notechis scutatus*) (*Med. J. Australia,* II, 1, 35). In the Western Hemisphere three Snake Venom Antitoxins are marketed.

Crotalus Antitoxin, know also as *Antivenin* (*Nearctic crotalidae*) and *North American Anti-Snake Bite Serum* is prepared from and effective against the venoms of the principal poisonous serpents of the Crotalidae. This includes the Rattlesnakes, the Copperhead and the Cotton-mouth Moccasin, the important poisonous snakes found in the nearctic region of North America, which includes Central and Northern Mexico, the United States and Canada, (Amaral, 1928, "*Newer Knowledge of Bact. and Imm.*, 1066).

Bothrops Antitoxin or ***Antivenin (Bothropic)*** or ***Tropical American Anti-Snake Bite Serum*** is prepared from and effective against the venoms of the principal poisonous serpents of the genus Bothrops, especially *B. atrox* (the Fer-de-Lance).

Antivenin Cascabel (Crotalus Terrificus) or *Tropical Rattler Anti-Snake-Bite Serum* is prepared from and effective against the venom of the tropical rattler, *Crotalus terrificus,* commonly known as the Cascavel or Cascabela, a poisonous snake which is widely distributed through the American tropics including northern South America, Central America and Mexico (except the north central highlands). The method of manufacturing marketing and employing these antivenins ("*Hand. der Path. Mikroorg.*" Kolle and Associates, III, 1) is with few modifications as given for North American anti-snake-bite serum which follows. The Bacteriological Institute at Buenos Aires issues a bivalent serum for *Crotalus terrificus* and *Bothrops alternata.*

NORTH AMERICAN ANTI-SNAKE-BITE SERUM

● In the preparation of *Antivenin* (*Nearctic crotalidae*) or *North American Anti-Snake-Bite Serum,* the several species of snakes are collected and the latter are made to imitate the natural "strike." The venoms are extracted by experienced workers employing these special techniques. They are then desiccated in an incubator. When ready to use the dried venom is dissolved in equal parts of saline solution and glycerin. It should be neutral (and *is* neutralized if necessary) and then placed in the refrigerator for one month to become sterile.

Animals are then immunized in the same manner as is done when making diphtheria antitoxin. Horses are used. Mules, cows and goats can be used when horses are not available. After immunization has progressed and the animal has been found to contain its maximum yield of antivenin, the first bleeding is conducted. The blood is then concentrated. The bleedings and concentration are practically the same as described under diphtheria anti-

toxin. North American Anti-Snake-Bite Serum, is, therefore, a purified and concentrated serum globulin to which is attached the antivenin obtained from horses which have been immunized against the venoms of poisonous North American snakes, belonging to the Crotalidae. Specifically it will antidote the poisons of the copperhead, the cotton-mouth moccasin, the rattlesnakes, etc., but it is not effective against coral-snake venom.

The usual precautionary measures as observed in preparing and marketing all biological products are also observed here. A potency test is always performed to be assured of the value of antivenin. The methods of standardization that are conducted consist in the use of any or all of the following tests: Precipitin test, neutralization test (neutralizing activity of venom) and the protection test. The latter is usually performed to determine the potency. This consists in injecting a 2-kilogram rabbit with 2 cc. of antivenin. No ill-effects should be observed after 1 mgm. of desiccated venom is injected two hours later. Pigeons and white mice are also employed as the test animals.

DOSAGE

● The antivenins are marketed in 10-cc syringes. This is an amount which will protect against the average quantity of venom secreted at one time by American serpents. The injection is given subcutaneously and better still intramuscularly as soon as possible. If too much time has elapsed between the bite and the arrival of antivenin, the latter is given intravenously. The preparation possesses excellent keeping qualities and is issued under a dating of many years (at least five years).

As soon as an injection is given, the tourniquet is released (*Bull. Antivenin Inst. Amer.* III, 43). Additional doses are given to children or where severe symptoms may have developed. The smaller the animal bitten, the larger is the dose of antivenin. It is rather fortunate that the North American pit-viper venoms are generally slow in exerting their effect, so that in most instances antivenin can be procured before death or even before marked serious effects are produced by the venom.

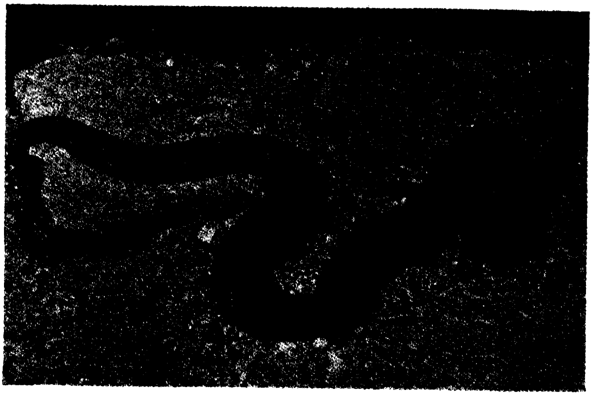
WHAT TO DO WHEN BITTEN

● When bitten by a snake, if possible, *kill or catch the snake that did the biting,* or if this is impossible, a careful or casual description of the snake may aid in the identification of the species. Knowledge of the fauna, and especially varieties of poisonous snakes apt to be present in any locality, is of great importance.

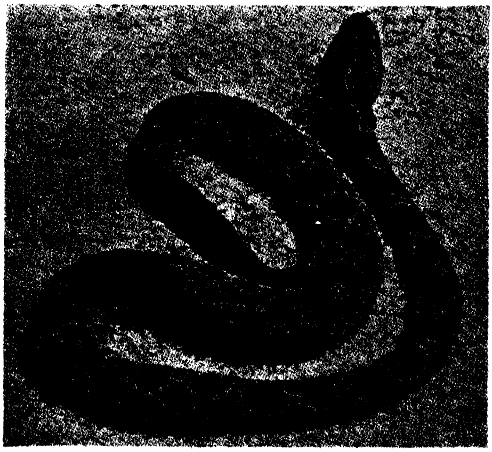
Just as it is necessary to capture a dog when bitten so as to find out if the animal has rabies, so one should attempt to find out whether the snake that did the biting is poisonous or non-poisonous. In the case of the snake, the identification is fortunately much easier than in the case of rabid animals. Such identification will enable one to avoid death or at least a considerable loss of time, discomfort, worry, money, etc. If the snake is a non-poisonous species, then no treatment is necessary, other than a local application of iodine or any similar disinfectant so as to prevent the slight wound from becoming infected.

If the snake that did the biting was a poisonous snake, then it might be necessary to identify the particular species. Just as in the treatment of gangrene we prepare and employ an antitoxin (antitoxic serum) which is specific for the neutralization of the toxin produced by the causative agent of gangrene; and in tetanus or lockjaw, tetanus antitoxin is employed to neutralize the toxin produced by the tetanus bacillus (which causes lock-

jaw), so specific antivenins (antivenomic sera) are necessary in the treatment of bites by venomous snakes. In other words, antivenin for Cobra venom is useful for the treatment of bites of Cobra but not other snakes. The antiserum or antivenin is specific for the treatment of the venom from which it was prepared. The tendency today is to prepare a polyvalent antivenin (one made from the venoms of different species of snakes) which will counteract the effects of all venoms from poisonous reptiles. It seems impractical or impossible to prepare a polyvalent antivenin specific for venoms of all poisonous serpents found in the world, but a polyvalent antiserum or antivenin specific for most of the poisonous reptiles in a given locality is possible and is available. An antivenin to counteract the effects of all poisonous snakes in Brazil is available, being



To the left, Agkistrodon mokasen, "Copperhead." Below, Sistrurus miliarius, "Pigmy or Ground Rattler."



produced by the Brazilian government at the Seropathic Institute of Butantan, near the City of Sao Paulo, in the state of Sao Paulo, Brazil. Dr. Amaral, through the Antivenin Institute of America, with headquarters in Philadelphia, has produced polyvalent antivenin which is effective against the venoms of the principal poisonous serpents of the Crotalidæ. This includes the Rattlesnakes, Copperhead and Moccasin (all pit vipers), the important poisonous snakes found in the nearctic region of North America, comprising Central Mexico, United States and Canada. Persons bitten by Coral Snakes found in the Southern States of our country and in Mexico would have to resort to other treatment. The so-called "Antivenin" (Nearctic Crotalidæ) or North American Anti-Snake-Bite Serum will not neutralize the venom of Coral Snakes. In other countries, antivenin specific for the venoms of the poisonous snakes in these localities must be employed.

If the snake is poisonous, there is also the possibility that the venom did not penetrate the circulation, or as in some instances there is the possibility that the poison glands are not functioning and venom cannot be secreted. In most cases, the presence of venom causes almost instant pain,

swelling, discoloration, etc., and the chances are that if these symptoms are absent, the individual was bitten by a non-poisonous snake. As a precautionary measure where the biting occurred in this country in areas where pit vipers prevail, one can take an injection of North American Anti-Snake-Bite Serum. This will not cause any outward effects, even though venoms from pit vipers are not present in the system of the individual into whom the antivenin is injected.

Every naturalist, botanist, prospector, hunter, and sportsman, tourist, perpetual hiker and camper, surveyors, civil engineers and workers for public utility companies and those engaged in forestry and construction operations venturing into regions known to contain venomous pit vipers should carry along with them first-aid treatment for bites by these poisonous snakes. The drug store or if there are no drug stores in small communities, then the general store and all camps in localities where these snakes abound should have at all times first-aid kits. In the latter the following articles should be found: Two packages of North American Anti-Snake-Bite Serum (or the specific antivenin if in other countries), containing sterile syringe and sterile needle; several stout rubber bands or a rubber ligature; a sharp knife, scalpel or razor, a few sterile bandages and a package of sterile gauze; a small breast pump or other suction device, and tincture of iodine or other antiseptic. Dr. Dudley Jackson and Colonel Crimmins, in their campaign against poison snakes in Texas, have been using effectively the Dudley Kit which contains small rubber bulbs, a unique suction type of apparatus. A first-aid outfit should be kept in a place where it can be obtained quickly, as promptness is essential in the beginning of the treatment.

(2) *Place a ligature or tourniquet,* preferably a rubber band, *above the bite (between the bite and the heart).* Don't lose time, but apply this promptly as soon as bitten, even if a strip of cloth torn from a garment has to be used instead of a rubber band, etc. By twisting a stick into the rubber band or ligature, the latter will be made tighter. The ligature should be released for a few seconds at five to ten-minute intervals to maintain necessary circulation and to prevent gangrene from setting in. This will prevent, or at least delay to a great extent, the systemic absorption of a deadly dose of venom from the amount present in the tissues immediately surrounding the wound. Above all KEEP YOUR HEAD, don't get despondent or excited, but ACT PROMPTLY AND SYSTEMATICALLY.

(3) If you are in reach of medical or scientific aid, call a doctor or a scientist who knows how to treat snake-bite wounds. If they cannot be reached, then get the proper serum, and inject it yourself. If you do not have antivenin with you, arrangements should be made AT ONCE to procure some. Telegraph and use the aeroplane if necessary. *The early judicious use of the proper antiserum or antivenin is the best remedy available today in the treatment of poisonous snake bites.* If you have to give the injection follow the directions as given on the label or circular. As the injection is about to be given, remove the ligature, or if the antivenin is administered within a few minutes after being bitten, a ligature or tourniquet is not needed, and in fact, should not be used, as it is necessary for the antivenin to circulate freely throughout the blood stream.

(4) Squeeze the wound to make the blood flow freely. This may carry away some of the poison. Incisions into and about the fang punctures so as to enlarge and make them deeper is still used by some but most workers today claim that there is no particular advantage in doing this. Suction

may be applied. It may prove to be of some value. Incision and suction are not necessary if antivenin is used early. In fact, if antivenin is available, incision is to be condemned, as there is a greater possibility of an infection setting in when the skin is cut.

(5) Don't pack the wound with crystals of potassium permanganate, and don't apply poultices or alum or parts of a snake's or other animal's body, or pour on the wound coal-oil or whiskey. Don't take alcohol or ammonia in any form internally.

Keep warm. Local treatment should be used by the Doctor, for it is of the greatest importance, as epsom salt or sodium citrate dressings for inflammation, novocain injections to alleviate pain, etc. Coffee (or caffein) or the use of strychnine should also be avoided as this tends to raise the blood pressure, but they may be used if symptoms of weakness or giddiness develop or if the fangs may have struck an artery so that the venom was injected directly into the blood vessel.

(6) There is always a possibility that a wound made by the bite of a snake may become infected. It is, therefore, advisable to apply an antiseptic or an antiseptic dressing to the wound, even though the individual bitten survives the primary effect of snake poisoning.

The use of alcohol internally is to be condemned, as it is undoubtedly harmful. Alcohol stimulates heart action, thus helping absorption and it lowers the blood pressure which is just what is not desired. In addition to the delay of the absorption of the poison, it is desirable to maintain at all times a high blood pressure. There is many a story told about whiskey having saved the life of a human who was bitten by a snake, and in practically all of these so-called "cures" by alcohol the biting was performed by a harmless snake.

SPIDER VENOM ANTITOXIN

● The Black Widow Spider (*Latrodectus mactans*) has been accused as the agent causing death due to the toxicity of its venom (*J. A. M. A.*, *86*, 1864 (1926)). Several fatalities have been reported in the western part of the United States. These spiders were once found only in rural districts, in grain bins, barns, chicken coops, grape arbors, in clusters of grapes, on tomatoes when removing them from the vine, etc., but within the past few years they have become extremely urban. Statements have been made that they are likely to become in the very near future a far greater menace than poisonous snakes. The spiders are extremely timid and never attack unless unwittingly disturbed or molested. Much experimental work is being conducted so as to make available an antitoxin or antiserum against black widow-spider-venom (*Proc. Soc. Exptl. Biol. Med.*, 1934, *32*, 166).

Lethal effects in man and animals from the bites of poisonous spiders in various parts of the world have been reported. The action of the venoms of such spiders has been studied carefully and antivenins have even been prepared. In Brazil, *Lycosa raptoria* and *Ctenus nigriventer* are responsible for most of the cases observed in Sao Paulo (*Bull. Antivenin Inst. Amer.*, 1928, II, 69). A bivalent antivenin for the venoms of the former is available (*Trop. Dis. Bull.*, 1927, XXIV, 402). Antiserum against venom of the black widow spider though produced successfully in sheep has been produced recently in rabbits. The rabbit serum compares favorably with the sheep serum and the use of the rabbit is advocated as being of greater commercial value (*Proc. Soc. Exptl. Biol.*, *40*, 686 (1939)).

SCORPION ANTIVENIN

● A specific serum against scorpion sting or venom is available (*J. Hyg.* IX, 69). Such a preparation prepared by the Lister Institute at Elstree, England, has been issued for use in Egypt. *Buthus quinquestriatus* is the common variety of scorpion in the latter country. In Brazil many deaths have been recorded due to scorpion stings and in particular from that of *Tityus bahiensis.* An antivenin effective for the venom of the latter and three other South American species is available (*Mem. Inst. Oswaldo Cruz.* XXI, 5). Scorpion antivenins in common with those derived from snakes and spiders are highly specific.

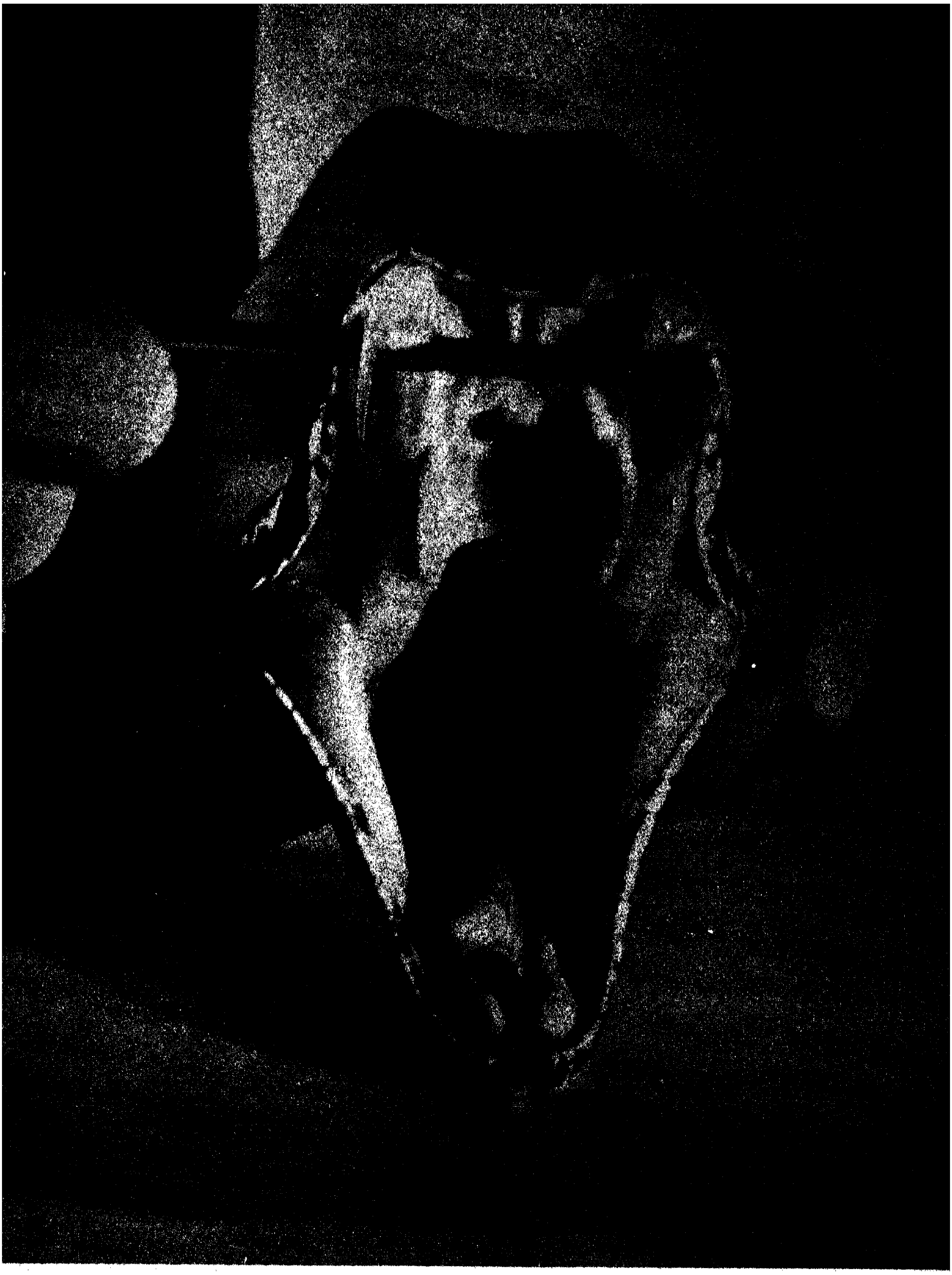
VENOM

● The snake venoms are viscous, yellowish liquids, clear or very slightly clouded; and containing in solution as they do an amount of protein equivalent to from one-quarter to one-third or more of their weight, they are grouped with the proteins. The remaining substances present are water, carbohydrates, small amounts of inorganic salts and occasional admixtures of abraded epithelial cells or other cellular material. Venom may contain different ferments or enzymes and one or more of several toxic elements which vary in their activity and action. Some of the ferments which may be found are: Proteases (proteolytic ferment), which may produce softening of muscle tissue; lipases (lipolytic ferment), which possesses a feeble action in the splitting of lecithin and in the fatty degeneration of the liver; a diastatic ferment, which may activate the inactive pancreatic juice so that the latter will attack albuminoids; and a fibrin ferment, which attacks fibrin, the coagulating element, resulting in a loss or reduction of capacity of the blood for coagulation.

SNAKE VENOM

The snake venoms contain one or more of several toxic elements which may attack red blood cells (classified as hemotoxins and hemagglutinins), nerve cells (neurotoxins), white blood cells or leukocytes (leukotoxins) and endothelium (classified as endotheliotoxin, endotheliolysin or hemorrhagin). The hemorrhagin is especially marked in the Rattlesnakes constituting the chief toxic element in the venoms of these pit vipers. The endothelial lining of the blood vessels, and in particular that of the capillaries, are destroyed and there soon appears hemorrhagic (bloody) infiltration and an enormous swelling around the wound. The venoms that are neurotoxic cause death by paralysis of cardiac and respiratory centers. Neurotoxins are present in but relatively small amounts in the venoms of the Pit Vipers but predominate, being present in great amounts and are the chief agents causing death in the venoms of the genus Micrurus containing the Coral Snakes as well as in the other Elapine Snakes, including the Cobras and their allies. The only important exception worth while mentioning is in the case of the South American Rattlesnake, which unlike other species of Rattlesnakes contains a strongly neurotoxic poison which has a selective action for the optic nerve, producing blindness almost immediately.

The action of most venoms in human beings is observed by a local reaction almost immediately after being bitten. These symptoms are usually severe and painful. The only important exception is in the case of bites by the Kraits found in the Old World, where in most cases local reactions do not occur. The poison or venom after being introduced into the deeper (subcutaneous) tissues, reaches finally the general circulation by absorption through the lymph and blood vessels. Snake venom is absorbed by the



View of open mouth of Rattler, poisonous snake found in United States, showing position of fangs. The venom is used in the preparation of Antivenin (Anti-snake-bite Serum).

Milking a poisonous snake of its venom to be used in the manufacture of Antivenin (Anti-snake-bite Serum).



conjunctiva and is not absorbed in the mouth or alimentary canal unless abrasions and cavities in teeth are present. The gastric juice destroys most all venoms except that secreted by the Cobra, the Old World Vipers, and the Black Snake of Australia (*Pseudechis porphyriacus*).

The rays of the spectrum, as well as rays from radium and electricity, reduce and finally destroy the toxic power of snake venom. Varying degrees of heat, differing somewhat for the venoms from the several groups of poisonous snakes, as well as certain chemicals, will detoxify venom *in vitro*. When snake venom is dried without heat, small amber-like crystals of material are obtained. Venom thus dried or desiccated retains the original toxic qualities of the liquid in unaltered strength for long periods of time.

THERAPEUTIC USE OF VENOM

● Attempts have been made to use the venom of snakes and other animals as therapeutic agents. Snake venoms have been employed for their hemostatic possibilities and possess a limited value in the treatment of tumors (*Lancet, 227,* 985 (1934); *Paris Med., 1,* 221 (1935) through *J.A.M.A., 104,* 1950 (1935)). They have not been made available commercially but supplies are obtainable for experimental use.

BEE VENOM

"Bee Venom" supplied in lyophilized form is marketed in vacules, each containing bee venom representing 10 stings. Injections are given preferably intradermally near the seat of pain. If this is not convenient, injections are given under the surface of the arms or legs. The dose is gradually increased until an injection of 1 sting is given. Multiple injections of 1 sting each are continued at intervals of 2 or 3 days. The total maximum dose at one treatment is 10 stings. Bee venom is employed in the treatment of acute and chronic arthritis and appears to be most valuable in the extra-articular manifestations (*N. Y. State Med. J., 38,* 1218, 1471 (1938); *Bee Venom Therapy,* Beck, N. Y. City (1935); (*Neb. Med. J., 24,* 298 (1939)).



Typing Pneumococci—Neufeld Technic

Equipment needed for rapid typing of pneumococci in sputum is shown above. Clean glass slides, cover-glasses, normal saline solution, a petri dish, Anti-pneumococcic Serum (rabbit) corresponding to each of the types for which therapeutic sera are now available, sample of sputum containing pneumococci, methylene blue solution and a platinum loop approximately four millimeters in diameter. In the above photograph the operator is using a capillary pipette to transfer typing serum to glass slides numbered to correspond to the type of diagnostic serum. A microscope, preferably equipped with oil-immersion lens, completes the list of necessary equipment.

In the above photograph the operator is using a platinum loop to transfer small flecks of sputum to each of the numbered glass slides. The loop need not be "flamed" between each transfer if the sputum is placed on the slide before the diagnostic serum. A drop of diagnostic serum of type corresponding to the number on the slide is added to each bit of sputum and mixed.. The loop must be carefully "flamed" between transfers to the slides to prevent mixing of serums. The mixture is "ringed" with petrolatum and covered (cover-glass) to prevent drying. A drop of methylene blue solution added to the mixture facilitates reading the test.

VIII

ANTIBACTERIAL SERUMS

THERAPEUTIC SERA

Antimicrobic Serums

THESE products are serums obtained from the blood of animals (usually horses) which have been immunized (actively) against particular bacteria and they contain antibodies that are specific against the bacteria used in the immunization of the animals. The bacteria employed are those which usually do not excrete a soluble toxin and accordingly the serums do not contain specific antitoxic bodies or antitoxin. The serums thus prepared are more complex in composition as well as in action. They depend for their efficiency upon one or more of the following antibodies: Agglutinins, precipitins, bacteriolysins or bactericidins, and opsonins or bacteriotropins. It may be that in some instances liberated endotoxin causes the production of some antiendotoxin which may in turn be found in some of the marketable serums. In addition to these immune serums of the lower animals (horses, cattle, goats, rabbits or oxen), immune human serum obtained from individuals convalescent from certain diseases are used at times in the prophylaxis and treatment of various affections.

ANIMALS FOR THE PRODUCTION OF BIOLOGIC PRODUCTS

● Animals used in the production of biologic products are kept under competent daily inspection and a preliminary quarantine for the period of at least one week before use. Careful examinations are conducted by a competent veterinarian. Only healthy animals free from disease are used. Animals of the *Equine* genus are tested for the absence of glanders (mallein test), and those of the *Bovine* genus must be shown to be free from tuberculosis (tuberculin test). All horses, except those which are actively immune to tetanus are given not less than 500 units of tetanus antitoxin semi-monthly or 2,000 units monthly. Stables must be well lighted and well ventilated and floors, stalls, etc., must be so constructed and cared for as to insure constant cleanliness. In case of actual or suspected hoof-and-mouth disease, glanders, tetanus, anthrax, or gas gangrene among animals used for the production of biologic products, the manufacturer must notify immediately the Surgeon General of the U. S. Public Health Service. Necropsy records are to be kept of all animals which die or are killed after having been used in the production of biologic products. All personnel, animals, and equipment used in the production of veterinary biologicals are to be kept separate from personnel, animals, and materials used in the marketing of biological products for human use.

PREPARATION OF ANTIBACTERIAL SERUMS

● The antibacterial or antimicrobic serums differ (as observed in the definition) from the antitoxins. The preparation of this class of products is in many ways similar to that employed in the production of antitoxins as: Using healthy horses, selecting and caring for the latter, the identical procedure in the collection of the blood after the animal has been immunized, the filtering of the serum, adding preservative and the performing of sterility and toxicity tests on the finished preparations. But herein lie the differences.

In the first place, the material that is injected subcutaneously into the animal is *not soluble extracellular toxins. The first injections consist of a culture or suspension of the specific organism which has been attenuated.* In the case of antipneumococcic serum it is attenuated pneumococci and preferably pneumococci that have been obtained from as many different sources as possible. In other words, the cultures that are used are inoculated with as many different strains of the bacterium, or the different individual strains are cultured and afterward mixed together. These are then sterilized in a water-bath at 60°C. for one-half hour to attenuate the bacteria. Using only one strain of a bacterium (commonly known as monovalent) will result in the production of a serum (known in this case as a monovalent serum) which will be of value specifically against the strain used, but which will probably possess little value against other strains. However, by using mixed strains (polyvalent), a product will result which will be of value against almost any strain of the particular bacterium.

Most all marketable antibacterial serums are polyvalent (i.e. prepared from mixed strains of the particular bacterium) unless otherwise labelled.

The horses are usually injected with these attenuated cultures for a period of from two to four months with increasing doses after each injection, depending upon how the animals react. At the end of this period, after a high resistance has been created, injections of small doses of a culture of living virulent mixed strains of the same bacterium are given. These injections with the live bacteria are continued, giving increasing doses at stated intervals until at about the end of from five to eight or even ten months after immunization was started the animal is ready to be bled. The time when to bleed, as in the case of the antitoxins, is solely dependent upon each individual animal. If after preliminary tests it has been found that the specific antibody content is at its maximum, and additional injections do not increase this antibody content, then the animal is ready for bleeding.

It has been found advisable in many instances to suspend the organisms in saline solution instead of using the broth cultures of these bacteria. If the latter are originally grown on a solid medium, then the growth can be washed down and thus suspended in the sterile saline. If liquid cultures are employed to obtain growth, the organisms can be thrown down by centrifugalization, washed with salt solution, and finally suspended in the latter, to be attenuated or immediately used, if practical, for injection.

With many bacteria, except *streptococci, pneumococci* and *Pasteurella (Bacillus) pestis,* it has been found possible to use living organisms from the start or shortly thereafter. Intravenous injections are most frequently given. But the actual mode of administration, dosage and frequency of the latter depend on the virulency or toxicity of the organism employed and the health and reaction of the horse. In the case of antiplague serum,

non-virulent strains of *Pasteurella* (*Bacillus*) *pestis* (the causative agent) are employed throughout the immunization, due to the dangers connected in handling the living organism.

The bleeding here is conducted in the same manner as is the bleeding of the horses in the manufacture of diphtheria antitoxin (which was considered previously). After the clotting of blood, which is collected in sterile containers, to which there is added some preservative, usually 0.25 to 0.4% cresol, the serum is obtained. The serum is usually allowed to stand a few weeks in a refrigerator until further separation of fibrin ceases. It is then filtered, if possible, through a Berkefeld or a similar filter, tested for sterility and toxicity, a preservative is added and the preparation is placed on the market for use. These finished products are the antibacterial serums.

POTENCY TESTS

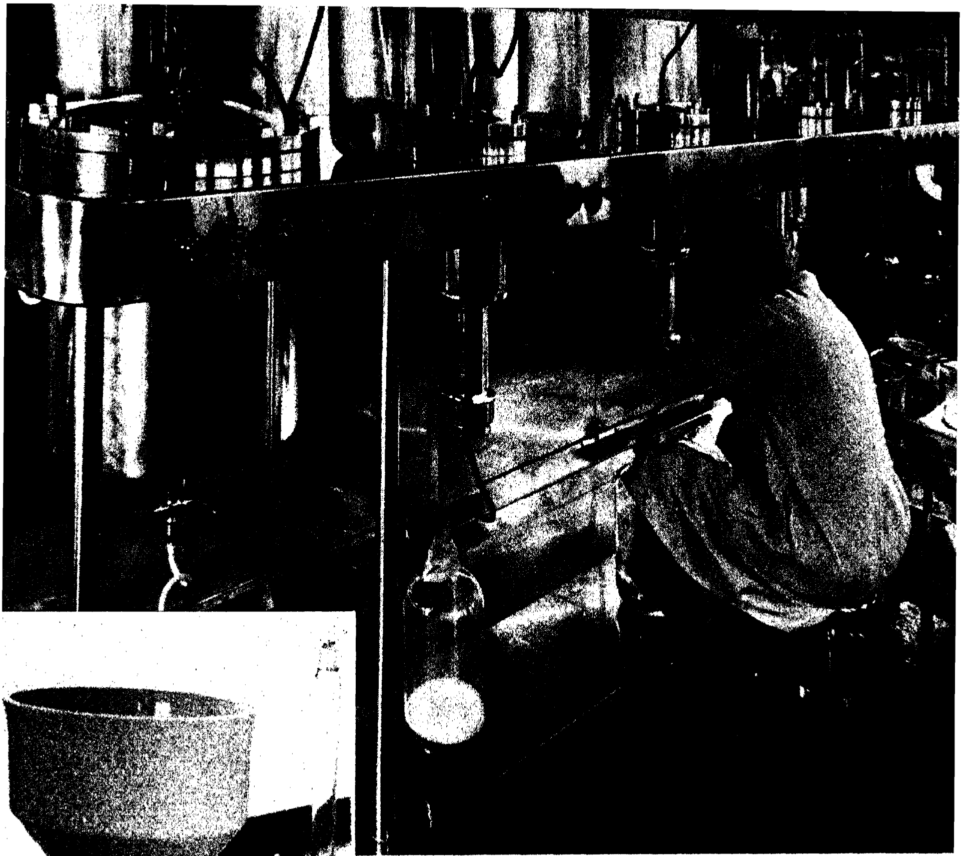
● As mentioned, the exact composition of the complex bodies which are present as the valuable antibodies, acting as specific therapeutic agents against the bacteria which produced them, is not known. On this account with but very few exceptions, it has been almost impossible to isolate them, or to remove the excess quantity of valueless and in many instances harmful blood serum (which is foreign to the human body), as is done in the manufacture of the antitoxins. For the same reason no satisfactory method has as yet been found by which the effectiveness or potency of these sera can be *accurately* determined, as is found possible in the case of the antitoxins. In the case of antipneumococcic serum, there has been established what may be called a protective and not a potency test. In addition to this, other biological tests are applied as agglutination and complement-fixation tests, to determine the content of agglutinins, complement-fixing antibodies, as well as others for different therapeutic sera, but none of them can be said to specifically inform one as to the exact potency of the particular product.

The following part of a committee report (Supplement, *Amer. J. Publ. Health, 28,* 93 (1937-38)) is of interest: "Although animal methods are generally employed in the testing of biologic products and although the protection of animals by a given serum or vaccine wears an aura of finality peculiarly satisfying to biologists, it is open to question whether some of our biologic methods of potency testing are quite as accurate as we believe. For this reason, we might well consider the future replacement of some of our biologic methods with chemical tests based on analysis of the specific precipitates found by antigens and antibodies mixed in certain proportions."

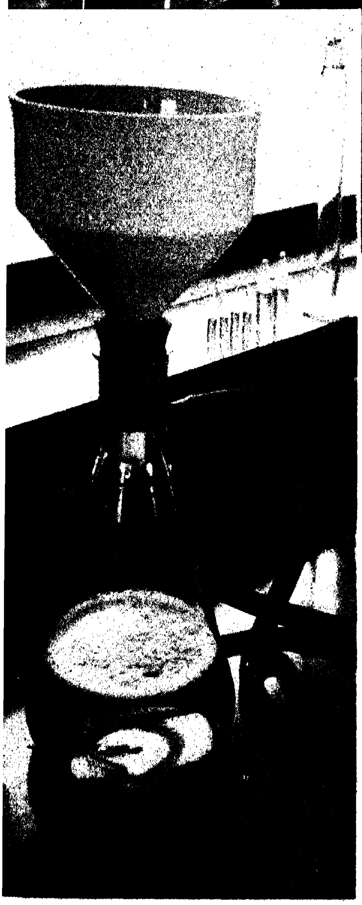
Due to the lack of definite standards and the uncertainty as to the promise of any beneficial effects, the antibacterial serums are not employed so extensively as therapeutic agents as are the antitoxins, though many therapeutic sera are marketed. Only serums having a high antibody content are marketed. At present the National Institute of Health requires official testing for only three antibacterial serums: Antipneumococcic, antimeningococcic and antidysenteric serums.

ADMINISTRATION

● The antibacterial serums are usually administered subcutaneously under the skin of the abdomen or between the scapulae. Intramuscular administration is gradually replacing the subcutaneous injections. Intravenous injections are employed in desperate or grave cases and in the late stages. Antimeningococcic serum is administered most frequently

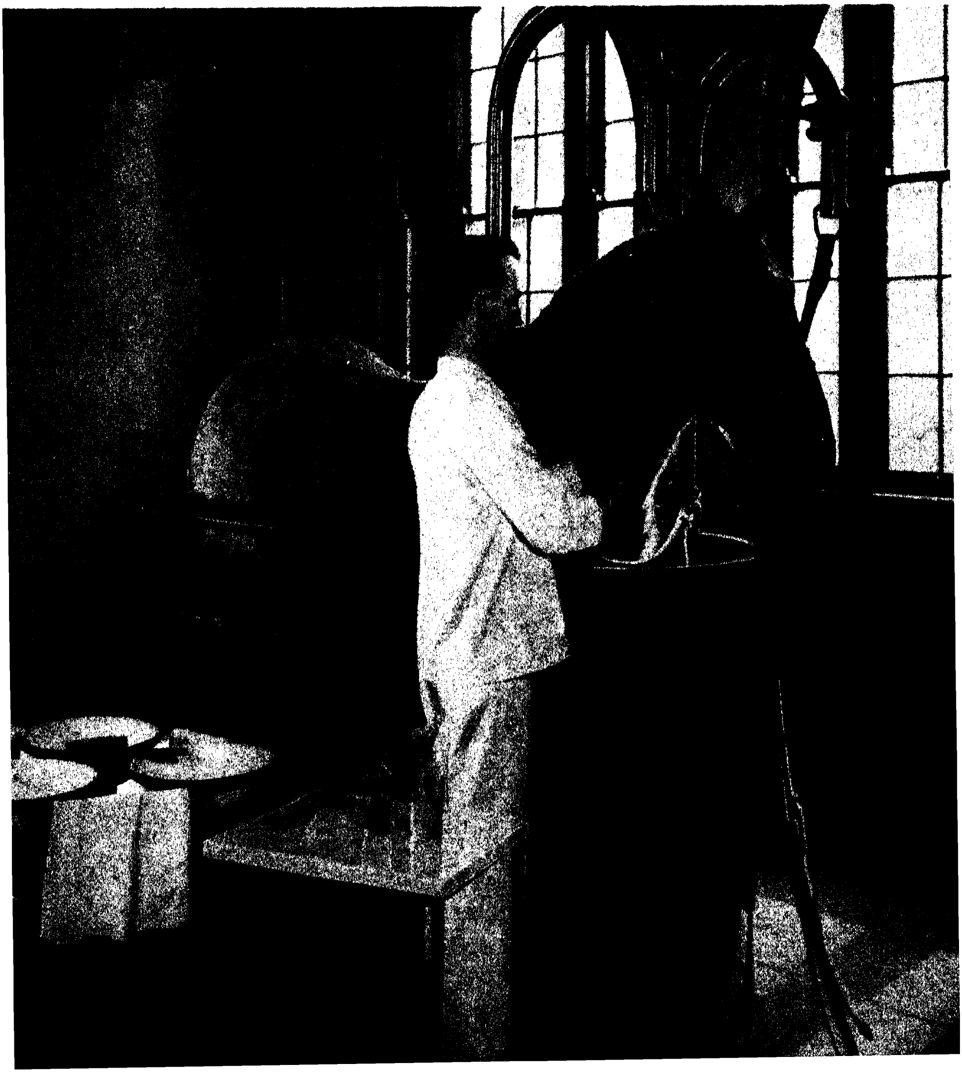


This battery of candle filters is used in the processing of sera.



Buchner filtration in the processing procedure in manufacture of biological products.

Giving an intravenous injection for the production of immune serum.



into the spinal canal (intraspinally). If this or any other serum is injected intraspinally, there is removed first an amount of cerebrospinal fluid equivalent to within 5 to 10 cc. of the quantity of serum that is to be injected, or until the pressure of the fluid is so reduced that only 3 to 4 drops flow out per minute.

In most all instances the minimum initial curative dose for adults is 30 cc. unless the serum is of a high potency. Injections are repeated with sufficient persistence until all symptoms disappear. This may be one or even two doses daily. Antimicrobic serums given intravenously or intraspinally are first warmed to body temperature and then administered very slowly. The safest procedure before using antimicrobic serums is to perform the skin test for sensitivity (see page 29). Regardless of the negative findings, some workers always give a preliminary subcutaneous dose of about 1 cc. of the serum approximately one-half hour before administration. This is a commonly used method of desensitization to avoid getting *serum sickness.* Those giving a positive skin test and so-called "horse asthmatics" (individuals who are sensitive to horse dander) require more elaborate methods of desensitization (as mentioned under Diphtheria Antitoxin). Including Serum Antipneumococcicum, Serum Antimeningococcicum and Serum Antidysentericum (Shiga), which will be considered individually, other antibacterial serums (which will be considered briefly) have been found beneficial or are being advocated as curative or prophylactic agents. They all should be employed as early as possible after the infection has been diagnosed and large doses should be given, and the serum used should be specific.

Before beginning the intravenous injection of a serum, it is a good practice to have at hand a hypodermic syringe containing 10 to 15 minims of a 1:1,000 solution of adrenalin (epinephrine). If symptoms of a reaction develop, the injection is discontinued immediately and the adrenalin administered. It is advisable to give the first injection of serum very slowly. The first cc. should require 5 minutes and the entire dose from 15 to 20 minutes.

VALUE OF ANTIMICROBIC SERUMS

● The value of these preparations has caused much controversy. They are given with the thought that they contain antibodies which are the same as the human body itself will produce if the patient overcomes the infection. Unfortunately infections get the upper hand before the resources of the body can begin their defense and produce protective substances. Antibodies produced in the blood stream of another animal and present in marketed serums serve the same function as if they had been produced by the patient. Such antibodies are made available almost immediately after they are introduced into the patient's system. They soon begin to diminish however. The immunity created therefore lasts rarely more than a few weeks. Obviously too, the inert proteins in the foreign serum may cause trouble by sensitizing the patient or by intoxicating an individual already sensitized. The value of the several marketed serums will be considered individually under their respective headings.

IX

SERUM ANTIMENINGOCOCCICUM

Antimeningoccic Serum

Serum Antimeningococ.—Antimeningococcus Serum, Meningococcus Serum, Meningitis Serum

"ANTIMENINGOCOCCIC SERUM is obtained from the blood of an animal of the genus *Equus* immunized with cultures of the several types of meningococci (*Neisseria intracellularis*) which prevail in the United States.

Description and Physical Properties

● A yellowish, clear, or slightly turbid liquid, having a faint odor of horse serum or having an odor due to the presence of a preservative; it may have a slight, granular deposit.

Antimeningococcic Serum must come from healthy animals and must be sterile. It must not contain an excessive proportion of preservative (not more than 0.35 per cent of phenol or cresol if either of these is used).

Antimeningococcic Serum must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Antimeningococcic Serum,* the manufacturer's lot number of the Serum, the name, address, and license number of the manufacturer, and a statement of the date beyond which the Serum may not be expected to retain the potency prescribed by governmental authority.

Storage

● Preserve Antimeningococcic Serum at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● Therapeutic, by parenteral injection, Metric, 20 cc."

UNTIL recently it was believed that meningococci were homogenous and that there were no essential differences between individual strains. Many workers have, however, revealed that there is extensive serological diversity among the meningococci. As a result of the researches of Gordon and associates (*"A System of Bacteriology in Relation to Medicine,* London, 1929, II, 312), four groups or types are recognized. Branham and coworkers (*U.S.P.H.S. Rep.* No. 1469, 1931,) reveal the relative proportions of meningococci which fall into these four groups as found in the United States. Recent work tends to show that the meningococcus possesses rough and

smooth variants (*J. Bact.*, 1932, xxiii, 93) (*Brit. J. Exp. Path.*, 1932, xiii, 380; 1933, xiv, 227). The former are likely to be less potent antigens than freshly isolated smooth strains. Wadsworth (*Trans. Assoc. Amer. Phys.*, 1932, *47*, 161) has reported good clinical results with a polyvalent serum made by the use of six representative strains, two each of types I, II, and III, which have been cultivated on laboratory media for almost 15 years. During epidemics of meningococcus meningitis, it is not difficult to obtain freshly isolated strains of meningococci but during inter-epidemic periods they are not always so easily obtainable. Murray (*Med. Res. Counc. Spec. Rep. Ser. No. 124*, London) and other workers found that the strains from sporadic cases were usually not typical. It, therefore, appears that antimeningococcus serums manufactured during non-epidemic periods unless prepared with care may possess little therapeutic value when fresh epidemics occur. It is with this in mind that in England the Ministry of Health (*"Review of Certain Aspects of the Control of Cerebrospinal Fever"*, 1931, London) conducted an inquiry into the results of serum treatment. Generally, however, the following technique is employed for the preparation of a polyvalent product.

PREPARATION

● In the preparation of marketable polyvalent antimeningococcus serum, horses that meet the usual rigid physical requirements are selected. Standard meningococcus strains, one or more from group I, group II, group III and group IV are grown separately on dextrose-serum-agar slants or other suitable culture medium. The growth preferably from 17 to 19 hour-old cultures is washed, the organisms are suspended in salt solution, and the suspensions are pooled (mixed). It is of interest to note that in preparing polyvalent antimeningococcus serum the selection of the strains of meningococci (*Neisseria intracellularis*) within the four types is limited to a small number. Each laboratory is allowed to decide which shall be used providing however that the strains employed possess wide antigenic properties. A few dominant strains covering the prevalent agglutinative varieties yield a more potent serum than is produced if many strains representative of all of the agglutinative variants are injected. The finished serum however must be potent against the more fixed or prevailing dominant types of meningococci associated with the meningococcus meningitis in the country where this preparation is to be used as a curative agent. Such a serum will be found generally to possess considerable curative power against the other less well-defined types found in that same area. In a recent study on meningococci isolated in the United States, Branham, Taft and Carlin found that at least 50 per cent of the 235 strains of meningococci isolated during 1928-30 were well agglutinated by the polyvalent sera prepared for therapeutic use by eight different manufacturers and 40 per cent were agglutinable but in less degree by most of these sera. The National Institute of Health supplies to manufacturers of biological products in the United States cultures of suitable strains of meningococci to be used for the preparation of a therapeutically efficient antimeningococcus serum to be employed in the treatment of meningococcus meningitis in this country.

The horses are immunized to the above prepared suspensions of meningococci which are either live or the first injections are attenuated or killed and the later injections are living organisms. With meningococci in particular, because of the toxicity of the autolytic products, as little delay as possible should occur between preparation of the suspension and its injection into

the animal. The suspensions are injected as soon as prepared. The injections are given either subcutaneously and in most instances intravenously into the jugular vein. Alternate subcutaneous injections of living organisms and an autolysate (*J. Exptl. Med., 10,* 141 (1908)) used formerly have been discontinued as it required from 10 to 12 months to produce a potent antiserum.

Intravenous injections establish more quickly a high degree of immunity, less of the culture or suspension of organisms is required, and certain complications are avoided (as the development of abscess formation). The first doses are very small (1/20 or less of an agar slant) and this amount is gradually increased. There is no definite schedule for dosage, this depending upon the reactions which develop. A daily injection is given on three consecutive days, these three injections being regarded as a series. Between each series, there is a rest period of seven days (*J. Exptl. Med., 23,* 403 (1916)).

This technique has been found the most rapid method for producing antiserum. Trial bleedings are made before the first and the fourth series of inoculations, and thereafter before every additional second series until the serum is found to possess the required potency. Sufficient potent serum is usually produced by most horses after about three months. When tests show the serum is of sufficient potency, whole bleedings (about 8 liters) are taken on the sixth day after the last injection. Inoculations are resumed on the fifth day after bleeding, and two or three series are given between each whole bleeding depending upon the potency. The trial and whole bleedings are made under aseptic conditions as described under diphtheria antitoxin, except that the whole blood is collected in flasks or jars and sterile citrate solution is not added: Large flat-wire egg beaters to support the clot are placed in the flasks after the latter are about half-full and the flasks are tilted. The blood is allowed to clot and the serum is drawn off with a siphon after twenty-four and forty-eight hours. Samples for sterility, toxicity and potency tests are taken and a preservative (tricrescol to 0.3% or other preservatives to a suitable concentration) is added. The bulk material is kept in the cold for several weeks and later it is filtered through paper-pulp filters and then through a Berkefeld filter, filled into sterile containers for distribution, and the filtered product is again tested for sterility before being issued. The expiration date is six months after date of manufacture or date of issue and storage is in the cold at temperatures between 2°C. and 10°C.

REFINED AND CONCENTRATED ANTIBACTERIAL SERA

● Various methods of refining and concentrating antisera are used (*J. Lab. and Clin. Med., 19,* 1324 (1934). The following procedure employed in the N. Y. City Dep't. of Health applies equally to the refining of pneumococcus and meningococcus antisera (*J. Lab. and Clin. Med., 24,* 417 (1939)):

"a. Preparation of Serum. Immediately after separation from the clot, 0.3 per cent phenol, in the form of a solution containing three part of phenol and five parts of ether, and 0.005 per cent merthiolate are added.

"b. Dialysis of Serum. Dialysis is carried out in a cold room at 7° to 10° C., the running tap water in the tank being at the same temperature. Dialysis is allowed to proceed for six days against a comparatively rapid flow of water. No additional pnenol is added during dialysis.

"c. 'Acid Protein' Precipitation. Immediately after dialysis solid sodium chloride is added to the cold serum to N/20, and the pH adjusted to 5.1 with normal acetic acid (γ-dinitrophenol as indicator). This is done in the late afternoon. The material is allowed to stand at 7° to 10° C. overnight. The removal of 'acid protein' by filtration through paper pulp and 'super cel' is carried out as before.

"d. Preliminary Tests for Antibacterial Substance Preparation. Four 50 ml. portions are adjusted with N/sodium hydroxide to the pH values 5.9, 6.1, 6.8, and 6.8. The first three are diluted with four volumes of distilled water while the last (pH 6.8) is diluted with six volumes. The

precipitates are allowed to settle in the cold, and are then centrifuged and dissolved in 10 ml. portions of 1 per cent sodium chloride solution. Supernatants and dissolved precipitates are tested by the precipitin reaction. The precipitate portions show potency and recovery of material; the supernatants, the optimum hydrogen ion concentration for bulk precipitation not incurring loss.

"e. Precipitation of Antibacterial Substance. No preservative is added to the distilled water used in the precipitation. The precipitated antibacterial substance is allowed to settle overnight in the cold, and finally centrifuged.

"f. Solution of Antibacterial Substance. The precipitate is dissolved in the smallest practicable volume of a solution of 2 per cent sodium chloride and 0.5 per cent phenol (about one-twentieth of the original volume as a rule). The final antibacterial solution is to contain 1 per cent sodium chloride and 0.35 per cent phenol. The above solution is, therefore, diluted with distilled water containing the calculated additional amounts of sodium chloride and phenol to give the required final volume as determined from the preliminary tests and the solid content desired (usually one-seventh to one-fifteenth of the original volume). The solution is adjusted to pH 6.8 with N/sodium hydroxide if the precipitating pH value was less than this, and 0.005 per cent merthiolate is added. The material is kept in the cold. After twenty-four hours, the preparation is centrifuged to eliminate the coarser particles of separated fibrin material. This procedure facilitates the subsequent filtration through paper pulp and 'super cel' (Buchner funnel) and the final passing through a small (6 inches) Berkefeld V filter.

"e1. Alternative Method of Precipitation of Antibacterial Substance. If an excessive amount of antibacterial substance is found in the supernatants of all the test portions, the material is not precipitated with ammonium sulfate to 50 per cent saturation as described in the previous paper. Instead, precipitation tests are run on portions of serum at the same hydrogen ion concentrations as before, but with the addition of six and eight volumes of water in place of four. This procedure almost invariably causes complete precipitation of antibacterial material with antibacterial-free supernatants. The bulk of the serum is adjusted to the determined hydrogen ion concentration, and the volume of distilled water needed for complete precipitation is added."

METHODS OF STANDARDIZING

● It is not possible to infect the ordinary laboratory animals and produce typical cerebrospinal meningitis. Neither are there produced in the serum of an immunized animal appreciable amounts of antitoxic or antiendotoxic substances which might serve to gauge with a fair degree of accuracy the potency of the finished antimeningococcus serum. An accurate method of standardizing this and other antibacterial sera as is possible with the antitoxins has not as yet been devised and it is, therefore, only possible to approximate the efficiency of therapeutic sera by indirect measures. Among the methods advocated are (a) Bacteriotropic titration, determining the opsonin or tropin content; (b) Determining the complement-fixation titer (*J. Exptl. Med., 10,* 141 (1908)); (c) Determining the protective value or power of the serum against mice treated with virulent cultures of meningococci (*Canad. Publ. Health J., 28,* 265 (1937)); and (d) By determining the agglutinin content (*J. Exptl. Med., 23,* 403 (1916)). The latter offers the best method and is the method of choice and the technique most frequently used for marketable polyvalent antimeningococcus serum. If the latter is found to possess an agglutinating titer of from 1:5000 to 1:1500 against the strains of meningococci injected into the animal, it is regarded as being acceptable as a potent marketable preparation. In standardization tests its agglutinative action is compared against the four homologous standard group strains of meningococci in comparison with the agglutinative activity of a standard control serum, the standard products being supplied by the National Institute of Health.

The statement of *"No U. S. Standard of Potency"* need not appear on the labels. Though there is no official standard, official tests are made at the National Institute of Health prior to the release of any product for sale in interstate commerce and in the District of Columbia.

USES

● The best results in the serum treatment of meningococcus meningitis, as with the use of other antibacterial serums, are secured in the early recognition of the case, avoidance of delay in injecting the specific serum, and the use of a potent preparation in sufficiently large doses as is compatible with safety. Intraspinal injection is employed when using antibacterial serums in the treatment of meningeal affections. As soon as the first suspi-

cion is aroused, a lumbar puncture should be performed. If meningococci are found in the spinal fluid which is collected in a sterile tube, antimeningococcus serum should be administered as quickly as possible. If the clinical symptoms point strongly to epidemic cerebrospinal or meningococcus meningitis, or even if the physician is only suspicious, if the fluid withdrawn is cloudy, or if direct smears made immediately reveal nothing definite, it is advisable to inject the serum without a further delay for additional bacteriological findings. Even if the latter are later reported as negative for meningococcus, no harm will have been done. The technique of serum injection consists of tapping the spinal canal, and allowing the fluid to drain (without suction) until the pressure is so reduced that only 3 to 4 drops escape per minute. The serum previously warmed (in a water-bath) to body temperature is then introduced slowly through the needle and preferably by gravity, an ordinary injection consuming at least fifteen minutes. The dosage is regulated according to age and the clinical symptoms of the patient. The average dose for infants and children is from 10 to 20 cc. and for adults from 20 to 40 cc. the volume of serum injected depending upon the amount of spinal fluid withdrawn, the former being always less by several cc. (5 to 10 cc. less) than the latter. Repetition of the injections is as important as the initial injection if a cure is to be effected. In mild or moderate cases, one injection daily will suffice. This may be increased in severe cases to two daily injections for the first day or two and then daily injections. Usually four to six daily intraspinal injections control the average case that is treated with sufficient promptness, but administration of serum should be continued until the spinal fluid is sterile. As soon as the latter condition prevails, it may be advisable to give one or two additional injections to guard against a relapse. Many workers control the withdrawal of spinal fluid and the injection of serum by blood pressure measurements as a fall of blood pressure usually occurs in both procedures. Though of value, this is not an absolute guide. During the withdrawal of spinal fluid, a drop in pressure of 10 mm. of mercury in adults and 5 mm. in children indicates that sufficient fluid has been withdrawn. During the intraspinal administration of the serum, a sudden drop in blood pressure should lead to caution and even an immediate cessation of injection, the latter to be resumed cautiously if the symptoms of collapse disappear in a few minutes. A total drop of 10 mm. in children or 20 mm. in adults during injection calls for a cessation of the latter. During epidemics and in severe fulminant cases, it is advisable to give an initial injection intravenously of from 30 to 100 cc. preceding the injection by the withdrawal of blood for blood culture. If the latter reveals a bacteriemia or if a persistent or recurring hemorrhagic rash occurs additional injections are given intravenously, being governed as to repetition by subsequent blood culture control. Some workers advocate the injection of a total large dose intravenously under all conditions with or without intraspinal therapy.

Naccari (*Pediatria, 46,* 509 (1938)) advocates treating patients with antiserums prepared with meningococci isolated from patients in the same city. He found that the recovery rate was twice as great as in patients treated with antiserums prepared with meningococci from other places. Bouldon (*J. Pediatr., 13,* 357 (1938)) in reporting on 94 cases of meningococcus meningitis treated at the Bellevue Hospital from 1928 to 1936 concludes that the intraspinal administration of a potent serum every 12 hours for the first few days gives best results. Cormack's observations (*African Med. J., 12,* 311 (1936)) reveal that a properly selected serum

produces a marked reduction of mortality. It may be important to point out that an effort should be made to culture the meningococci in the infected spinal fluid and ascertain if the organisms are agglutinated by the antiserum used. If not, obtain and use an antiserum which will cause agglutination of the meningococci isolated.

The therapy of meningococcus meningitis as well as that caused by other organisms is being made more effective by the introduction of sulfanilamide, sulfapyridine and related compounds. It is possible that the latter will replace serum therapy or that they will be used in combination with antisera.

VALUE

● Great success appears to have attended the use of antimeningococcus serum when properly employed. In the majority of cases the subdural injection of a potent serum in meningococcus meningitis is followed by some immediate improvement. Its curative powers depend upon its action in destroying the meningococci and the small amount of antitoxins and anti-endotoxins neutralize the toxic agents produced by some strain of the latter. Due to the presence of bacteriotropins (*immune opsonins*), bactericidins, agglutinins and anti-aggressins, multiplication of the organism is arrested and phagocytosis and intracellular leukocytic digestion are accelerated. Before the introduction of specific serum therapy the mortality from meningococcus meningitis varied from 70 to 90 per cent. The mortality of serum-treated cases averages 25 per cent, generally does not exceed 30 per cent, and, if treated early, the mortality falls before 20 per cent, and usually is about 18 per cent. It is significant to note that there has been a yearly rise in the number of meningococcus meningitis cases in the United States though the actual number is not large when compared with the total population. The most important measures to be considered in the prevention and control of this infection especially when epidemics prevail are: (1) Prompt recognition of cases and reporting same to health authorities; (2) Immediate use of therapeutic sera; (3) Sterilization of dishes and eating utensils; (4) Maintenance of high standards of bodily vigor for everyone and the introduction of measures for a general improvement of the respiratory sick rate which is usually high during such epidemics; (5) Avoidance of public gatherings and overcrowding; (6) Detection and isolation of carriers, focusing the carrier examinations upon the small epidemiologically determined intimate group from which the case arose; and (7) Optimum of fresh air and sunshine for carriers and convalescents.

MENINGOCOCCUS ANTITOXIN

● In 1931, Ferry and associates (*J. Immunology,* XXI, 293) revealed that bacteria-free filtrates of the four Gordon types of the meningococcus contain specific soluble or extracellular toxins. These same workers (*J. Immun.,* 1932, XXIII, 315, 325; 1934, XXVI, 133, 134) conducted extensive researches on the production and value of a meningococcus antitoxin. Intravenous, intraspinal and intramuscular injections of such a preparation have yielded favorable results and have been proved by clinical trial to be efficient for the treatment of meningococcic meningitis (Hoyne, A.L., *J.A.M.A., 104:* 980, (1935)). This preparation is marketed and the Council on Pharmacy and Chemistry (*J.A.M.A., 104:* 1007, (1935)) accepted a meningococcus antitoxin for a trial period of one year or more or until a clearer evaluation of its usefulness was obtained. Horses are immunized with polyvalent filtrates of 6-8 day hormone-broth cultures of the 4 Gordon

groups of (types I, II, III, and IV) meningococcus, after the method of Ferry, Norton and Steele. The dosage for humans is 5,000-10,000 units of antitoxin injected intraspinally, intracisternally or intravenously at 12-24 hour intervals. Hoyne (*J.A.M.A., 107,* 478 (1936)) has advocated the exclusive use of intravenous therapy as the method of choice. For further reports on Meningococcus Antitoxin see *Kentucky M.J., 35,* 180, 464, (1937). The average case requires 50,000 to 100,000 units. One unit is 10 times the amount of antitoxin, which when mixed with 1 skin test dose of meningococcus toxin produces a negative cutaneous reaction. A skin test dose of meningococcus toxin is an amount which injected intradermally into a susceptible person produces a cutaneous reaction of at least 10 mm. diameter. The marketed meningococcus antitoxin contains 10,000 units. Many workers are of the opinion that the so-called exotoxin is in reality an endotoxin as the meningococcus is susceptible to spontaneous autolysis. They believe that Ferry's serum is not a true antitoxin but that it does possess therapeutic activity due to antibacterial and antiendotoxic antibodies (*J. Immunol., 23,* 291 (1932); *33,* 375 (1937); *J. A. M. A., 107,* 478, (1936)). For further considerations see *J. Lab. and Clin. Med., 23,* 252 (1937).

COMBINED TREATMENT

● The combined therapy of antisera or antitoxins with other chemotherapeutic agents is being used more frequently in many affections. Jernigan (*J. Arkansas M. Soc., 35,* 40 (1938)) recommends for the treatment of cerebrospinal meningitis the intraspinal administration of the antimeningococcus serum and the intravenous administration of meningococcus antitoxin. Norbury (*Military Surg., 83,* 76 (1938)) suggests the combined therapy of the antitoxin and sulfanilamide. Muraz and Associates (*Presse Med., 46,* 1113 (1938)) discuss comparative methods of treatment with antisera and other chemotherapeutic agents.

MENINGOCOCCUS VACCINE

● Meningococcus vaccine (stock suspensions of attenuated meningococci) has been and is being used. Three injections at weekly intervals of one-quarter, one half and one billion organisms respectively have been reported as giving rise to considerably antibody production and are recommended as a prophylactic treatment during unusual prevalence of the disease. Favorable results have also been reported with autogenous vaccines in the treatment of meningococcus meningitis when long continued serum therapy has failed to cause complete recovery. Injections of these autogenous preparations are given at two or three day intervals in doses ranging from one-tenth to one billion or more bacteria. The exact value of meningococcus vaccine is to be determined, as the statistics are too few to be conclusive. Indications point to its value at least as a prophylactic agent during severe epidemics, especially if the vaccine is prepared from a freshly isolated virulent strain (*J. Immunol. 33,* 51 (1937)).

X

SERUM ANTIPNEUMOCOCCICUM

Antipneumococcic Serum

Serum Antipneumococ.—Antipneumococcus Serum, Pneumonia Serum

"Antipneumococcic Serum is obtained from the blood of an animal of the genus *Equus*, or of the genus *Lepus*, which has been immunized with cultures of a pneumococcus (*Diplococcus pneumoniæ*) of one of the varieties known as type I, type II, type IV, type V, type VII, or type VIII.

Description and Physical Properties

● A yellowish, clear, opalescent, or slightly turbid liquid, having a faint odor of serum or having an odor due to the presence of a preservative. It may have a slight granular deposit.

Antipneumococcic Serum must come from healthy animals, and must be sterile. It must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol, or 0.4 per cent of cresol if either of these is used).

Antipneumococcic Serum must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Antipneumococcic Serum* and the specific type of pneumococcus represented, the species of animal employed, the manufacturer's lot number of the Serum, the name, address, and license number of the manufacturer, and a statement of the date beyond which the Serum may not be expected to retain the potency prescribed by governmental authority.

Storage

● Preserve Antipneumococcic Serum at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● Therapeutic, by parenteral injection, Metric, 30 cc."

THE classification of pneumococci based upon the agglutination reaction into three fixed types, designated as I, II, and III and a miscellaneous group IV was widely accepted and has been in use for a period of time covering almost a quarter of a century. For many years group IV was regarded as consisting of pneumococci, which because of their heterogeneity appeared to be unclassifiable by immunological methods. Subsequent investigations

resulted in the extension of the serological differentiation of group IV, and 29 additional types were classified as being in this heterogenous 'Group IV'. At present 32 separate and distinct serological types of pneumococci, designated as types I to XXXII are recognized. Recent investigations point to the fact that types XXVI and XXX are identical apparently with types VI and XV respectively. It may be that in the near future pneumococci will be segregated into 30 instead of 32 separate and distinct serological types.

Approximately 55 to 60% of the cases of lobar pneumonia are caused by pneumococci types I and II; 9 to 10% by type III; 25 to 30% by types in group IV, and 5% by organisms other than the pneumococcus. The mortality rate for the types of pneumococci is approximately: 25 to 30% in types I and II; 45% in type III; and 15% for the types in group IV. Pneumococci found in normal throats or in slight inflammatory conditions are observed usually (in more than two thirds of all cases) to be heterogenous strains of miscellaneous group IV, and in over 30%, type III may be found.

TREATMENT

● In the treatment of pneumonia, favorable results have been reported by various drugs, including dyes, optochin, oxygen, Vitamin C, deuterproteose, quinine and cinchona compounds, serum, diathermy and other therapeutic measures. Sulfanilamide and related products have been used in type III pneumococcus infections. It has been recognized for some time that success with serum treatment depends upon an early diagnosis and the immediate use of large doses of specific type serum. Though it has been difficult to assess the value of serum therapy in pneumonia, most observers agree that type I serum is beneficial. Others have reported good results in the specific treatment of type II pneumonia. Some States are supplying serum for use in type I and type II cases. More recently favorable reports have been presented on the use of specific sera in the treatment of pneumococcus pneumococcus rabbit serum as a therapeutic agent in lobar pneumonia has VII, VIII, IX, and XIV. The greater effectiveness of type specific antipneumococcus rabbit serum as a therapeutic agent in lobar pneumonia has been reported by many workers. It is possible to administer rabbit serum more rapidly, in larger amounts and in fewer doses than horse serum. More details are given in the following pages.

TYPING PNEUMOCOCCI *(See also page 65)*

● Treatment with specific type serum has resulted in the necessity of typing all pneumonia cases. Many techniques have been introduced for typing pneumonia sputum, but the Neufeld (Quellen) Method (*Preventive Med.*, *7*, 39, 1937)) has been widely acclaimed because of its simplicity, accuracy, and ease in obtaining a quick report. In this method, a loopful of the sputum is mixed separately with an equal amount of each of the available specific type serums (rabbit) and an equal quantity of Loeffler's Methylene Blue. After 2 or 3 minutes, they are examined. When the organism is the same type as the serum, the capsule of the pneumococcus swells to several times its usual thickness, thereby identifying the type. In practice, the sputum is most frequently tested only with diagnostic sera types for which specific serum therapy is possible and has proven of value. Pneumococcus types causing lobar pneumonia vary from year to year, making it difficult for health laboratories to have available adequate amounts of different type serums. Though type I pneumococcus is most prevalent and most frequently occupies the number one position, second place has been held at different

occasions by types III, VII and VIII. Bullowa (*The Management of Pneumonias*, Oxford Univ. Press) and Finland (*Arch. Path.*, *23*, June 1937) report that in typical pneumonias in adults, types I, III, II, V, VII, VIII and IV pneumococci accounted for most of the cases and in atypical pneumonia of pneumococcus origin, type III predominates, followed in order of frequency by types VIII, V, I, VII, VI, XX, XXVIII, and II. According to Bullowa, pneumococcus pneumonias in children are due to the following types and in the following order as to frequency, XIV, I, VI, X, XIX, IV, III, VIII, and VII. He states that types III, IV, V, and VII attack children of all ages. Types XIX and VIII attack only children under six years of age and type XIV attacks children mainly under six. In a report of many thousand cases (*Amer. J. Publ. Health*, *27*, Feb. 1937) the type distribution in lobar pneumonia for adults is quoted as 29% for type I; 17% for II; 12% for III; 5% for IV; 7% for V; 2% for VI; 6% for VII; 8% for VIII; 3% for XIV; and 11% for all other types. The type distribution in children is markedly different. The incidence of types II and III in children is very low. Type I occurs infrequently as compared with adults. Types VI, VII, XII, XIV and XIX are most frequently encountered in children having pneumococcus pneumonia.

THE OFFICIAL U.S.P. SERUM ANTIPNEUMOCOCCICUM

● In the U.S.P., there is official Serum Antipneumococcicum. This is a univalent or monovalent type antipneumococcus serum and is specific in its protective power only against the specific type represented. In the production of this serum, strains of all polyvalent types of the organism are not selected as in the case when preparing antimeningococcus serum and other therapeutic sera. Antipneumococcus Serum U.S.P. is prepared from the serum of horses highly immunized against either type I, type II, type IV, type V, type VII or type VIII pneumococcus. Standard type strains can be obtained from the National Institute of Health. The production of antipneumococcus serum with specific pneumococcus types as a starting point corresponds closely with those procedures described under the manufacture of Antimeningococcus Serum. Rabbits instead of horses may be used for immunization.

METHODS OF STANDARDIZATION

● For this as well as all other therapeutic sera prepared from pneumococci, the potency or titer of the marketable serum for therapeutic use is gauged by testing its protective value for white mice against a culture of the homologous standard type strain, as compared with the protection afforded by a standard control serum (supplied by the National Institute of Health) representing the minimum standard of potency. The National Institute for Medical Research in London has prepared and issues a stable standard control serum for manufacturers in Great Britain (*Lancet*, 1933, II, 91). Standardization by mouse protection experiments, (Park and Williams, *Pathogenic Microorganisms*, 1933, p. 292), though not entirely satisfactory as a test of the curative value of the preparation has been however, generally accepted as the most reliable method for determining pneumococcus serum potency. Precipitation (*J. Path. Bact.*, 1932, XXXV, 509) and agglutination titers of the serum though of value as partial indicators of the protective and therapeutic value of the preparation have not received as yet general recognition as acceptable methods for standardizing antipneumococcus serum. The specific polysaccharide precipitation test devised by Zozaya, Boyer, and Clark (*J. Exp. Med.*, 1930, *52*, 1471) is, however, advocated by some workers as of practical worth in establishing the compara-

tive value of a test serum to a standard serum. For a comprehensive study of standardization techniques see Felton and Stohl (*Nat. Inst. Health Bull.* No. 169, Feb. 1937). In the "Protection Test", it is important to be assured that the culture is highly virulent or at least as virulent as possible for the test animal and finally that it is of an accurately known virulence.

STANDARDIZATION

● In the case of the standardization of antipneumococcus serum, the test animal is the mouse (weighing approximately 20 grams (18 to 22 grams)), and the virulence of the pneumococcus used should be so great that 0.00,000,1 cc. or less of a sixteen hour (or 0.00,000,001 cc. of a 3 to 6 hour) broth culture will kill a mouse in forty-eight hours. The virulency of a pneumococcus if not high can be raised by animal passage. Dilutions of the broth culture of the virulent pneumococcus used to determine virulency are prepared in such a way that 0.5 cc. of each dilution contains varying quantities of the original pneumococcus culture ranging from 0.1 cc. or 0.2 cc. to 0.00,000,01 cc. and even 0.00,000,000,1 cc. To each 0.1 cc. of pneumococcus culture, 0.2 cc. of the antipneumococcus serum to be tested (diluted with saline solution to 0.5 cc.) is added and the mixtures are immediately injected separately intraperitoneally into (20 gm.) mice, preferably into the abdominal wall just above the groin. For each serum tested at least 3 mice are injected. The 0.2 cc. of serum to be tested can be injected and then followed in less than 5 minutes by 0.1 cc. of culture. In the tests, control and comparison tests are carried out. Two virulence control mice are inoculated with the dilution of the culture only, and a series of mice are injected with the standard serum (supplied by the National Institute of Health) and varying amounts of culture. A somewhat different method of standardization was introduced by Felton. In his technique of standardization, Felton employs varying quantities of plain or refined and concentrated antipneumococcus serum and a constant number of virulent pneumococci. A unit of antipneumococcus serum (plain or concentrated) as the term introduced by Felton and accepted today as a fundamental unit of potency for the latter, is ten times the smallest amount of antiserum which will protect the majority of (20 gm.) mice inoculated intraperitoneally with 100,000 fatal doses of a fully virulent culture of *Diplococcus pneumoniae.* In other words, this provisional unit standard in general use is the amount of antiserum which will protect a (20 gm.) mouse against (at least) one million M.L.D.'s or fatal doses of a pneumococcus culture. An M.L.D. here is regarded as the smallest amount of a pneumococcus culture which will kill a 20 gram mouse (when injected intraperitoneally) in forty-eight hours.

Goodner and Associates (*J. Bact., 36,* 276 (1938)) point out the objections to the mouse protection system for evaluating therapeutic antipneumococcal sera, that (1) there is no reference of potency from serum of one type to another, and (2) the test is rather expensive. They advocate the use of a modified technique of the immuno-chemical procedure of Heidelberger, Sia and Kendall.

The statement "No U. S. Standard of Potency" need not appear on labels. Though there is no official standard, official tests are made at the National Institute of Health prior to the release of marketable products.

OTHER MARKETED ANTIPNEUMOCOCCIC SERUMS

● In addition to Antipneumococcus Serum, Type I, a bivalent (mixed) antipneumococcic serum is marketed as ***Antipneumococcus Serum, Types I and II*** (*Am. J. Med. Sc.,* June, 1931, p. 788). The latter is equivalent to

the Type I Serum in its protective power against pneumococcus type I, but it also contains antibodies specific for pneumococcus type II. This combined serum is just as efficient as Type I Serum for use in treating pneumonia caused by pneumococcus type I, and possessing agglutinins specific for pneumococcus type II, it has the additional advantage of being able to possibly afford some protection against infections caused by the latter organism (type II). The National Institute of Health passes for distribution Type I and Type II serums providing they show at least 1,000 units per cc. if concentrated and at least 500 units per cc. if not concentrated. A Polyvalent Antipneumococcus Serum (Types I, II and III) is also available. Bivalent or polyvalent horse antisera for other closely related types are being produced, especially types IV and VIII, V and VII, VI and XIV.

Monovalent antipneumococcic rabbit therapeutic sera for most all types (I to XXXII) are marketed. Rabbits are immunized rapidly so that it has been found practicable to produce antisera for those types having a low incidence rate and also an antiserum for type III. It has been impossible to produce a clinically effective type III horse antiserum but rabbits have been found capable of producing such an efficient antiserum. Many reports have been presented recently in which antipneumococcus rabbit serum rather than horse serum is advocated for treating type specific pneumococcus infection and especially infections of the "higher types." Of interest is the recent warning by Hoagland and Associates (*Science*, Sept. 16, 1938) that rabbits' rather than horses' antisera should be used for treating type XIV pneumococcus pneumonia. They point out that the polysaccharide from type XIV pneumococcus is closely related, chemically and immunologically to the blood group specific substances as to possibly unite in the horse but not in rabbits' antibodies which will cause the agglutination of human erythrocytes.

CONCENTRATES

● It has been known for a long time that antibody combined with its antigen (the agent responsible for the development of the antibody) can be dissociated to a greater or less extent by the use of appropriate extractives. A method of obtaining such antibody in solution free from nearly all of the serum proteins was announced by Huntoon (*J. Immunol.*, 1921, VI, 117). Such a preparation is marketed as ***"Pneumococcus Antibody Solution" (Types I and II Combined)*** and appears as a clear colorless solution. The actual preparation consists in mixing the immune serum (prepared in the usual way) with an equal volume of a heavy suspension of the specific organism. The mixture is incubated for one hour at 37°C. and then centrifugalized. The sediment is washed well with saline to get rid of any adhering serum. The antibodies are then dissociated from the antigen (in this case pneumococci) by emulsifying the sediment in salt solution containing 0.25 per cent sodium bicarbonate. The mixture is heated to 55°C. for one hour and then centrifugalized. The supernatant fluid containing the antibodies in solution is filtered through a Berkefeld filter and filled into sterile containers. Sterility and toxicity tests are performed in the usual way.

From experimental studies it was found that most if not all of the protective or curative antibodies in antipneumococcus serum precipitate with the globulin fraction. Concentrated globulins by refining antipneumococcus serum globulins were obtained but were not thought to be sufficiently potent for use in practical therapy.

An advance was made, however, by the method of concentration and refinement as recommended by Felton (*J.A.M.A.*, (1930), *94*, 1893); (*J. Immunol.*,

21, 357 (1931)). In his technique, practically all of the mouse protecting substance is precipitated from the antipneumococcus serum by treatment with a sodium sulfate solution. The globulin precipitate is collected and dialyzed until free of the sulfate. The dialysate is slightly acidified and filtered. Five volumes of water are added to the filtrate, the resulting precipitate (containing the protective antibodies) is collected, dissolved in tricresolated saline solution (or saline containing other suitable preservative) and the solution is then sterilized by Berkefeld filtration. Other methods of concentration in which alcohol or other precipitant is used are practiced. Sterility, toxicity and potency tests are performed in the usual way on the finished product. Monovalent and polyvalent mixtures concentrated by the Felton method are marketed and known as ***"Refined and Concentrated Antipneumococcic Serum"*** or ***"Pneumococcus Antibody Globulin"***, either Type I, or Type I and II combined. The usual marketable package of Concentrated Antipneumococcic Serum Types I, or Types I and II combined. contain either 10,000 units or 20,000 units of each type per dose. Pneumococcus Antibody Globulin Types I, II and III Combined contains per dose the same amount of units of type I and type II antibodies but a negligible quantity of type III antibody. Concentrated and refined antipneumococcic serums do not approach the perfection of concentrated and refined diphtheria and tetanus antitoxic serums. The expiration date of all antipneumococcic serum preparations is one year after date of manufacture or date of issue and storage is in the cold between 2°C. and 10°C.

TYPE SPECIFIC ANTIPNEUMOCOCCUS RABBIT SERUM

It is generally regarded that antipneumococcic horse serum is unsatisfactory for use in the Neufeld Quellung Reaction because of non-specific effects produced by such serum, (*J. Exp. Med., 57*, 139, January (1933)). Others have reported that if the serum possesses an agglutinative titer of 1:160 to 1:320, either immune horse serum or immune rabbit serum is equally reliable, (Lancet. *I*, 290, (1934)). Immune horse serum is likely however to give irregular reactions. Immune rabbit serum is highly type specific and does not give cross reactions. Due to this fact and also inasmuch as relatively small amounts of serum are required for the Neufeld test, antipneumococcic rabbit serum is used as the diagnostic agent in the microscopic (Quellung) test. In techniques where macroscopic agglutination or precipitation procedures are carried out diagnostic antipneumococcic horse serum may be used, this being more economical.

Diagnostic antipneumococcic rabbit sera are marketed to be used for the rapid typing of pneumococci by the Neufeld method. Each of the types are separately available and to facilitate segregation of the types, various combinations are supplied as Group A containing types I, II and VII; Group B containing types III, IV, V, VI and VIII; Group C containing types IX, XII, XIV, XV and XVII; Group D containing types X, XI, XIII, XX, XXII and XXIV; Group E containing types XVI, XVIII, XIX, XXI and XXVIII and Group F containing XXIII, XXV, XXVII, XXIX, XXX, XXXI and XXXII. Type XXVI is identical apparently with type VI and type XXX with type XV. Combinations of type-specific sera varying from the list as given previously are also marketed.

PREPARATION OF DIAGNOSTIC ANTIPNEUMOCOCCIC RABBIT SERUM

Various methods are used for producing Diagnostic Antipneumococcic Rabbit Serum. Cooper and Walter (Amer. J. Public Health, April, 1935) present the methods used at the New York City Department of Health

Laboratories. They stress the need for selecting representative strains of pneumococci for immunization and the use of clean syringes for giving the injections. White (Biology of Pneumococcus, Commonwealth Fund, New York City, 1938, Appendix, page 663) gives the following technique as the:

Massachusetts Antitoxin and Vaccine Laboratory Methods

a. Vaccine. Eighteen hour broth cultures of highly virulent, type-specific pneumococci are centrifuged, the sediment taken up in physiological salt solution in quantities to give a corrected Gates reading of 2.0, and the suspension heated for 15 minutes at 100°. This results in a suspension containing approximately 25 billion organisms per cubic centimeter and is used as a stock supply of vaccine. No preservative is added. For the injection of rabbits the stock vaccine is diluted 1 to 10 with physiological saline solution, thus giving a suspension containing about 2.5 billion organisms per cubic centimeter.

b. Injection of rabbits. A minimal number of six rabbits should be maintained on each type of vaccine. All injections are given intravenously, the schedule and dosages following the scheme given below:—

Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1	0.1	0.2	0.5			
2			0.2	0.5	1.0	
3	REST					
4	0.5	1.0	2.0			
5			1.0	2.0	3.0	
6	REST					
7	1.0	2.0	3.0			
8			1.0	2.0	3.0	
9	REST					
10					BLEED	
11	1.0	2.0	3.0			
12			1.0	2.0	3.0	
13	REST					
14					BLEED	

c. Bleeding of rabbits. The rabbits are bled by cardiac puncture every three weeks after routine bleedings are initiated and, with careful handling, the animals may be kept for an average of at least a year. It has been found advisable to have available a supply of buffered glucose solution to administer intravenously to the occasional animal suffering severe systemic shock after having been bled. A minimum of 20 cc. of blood is removed from each rabbit and placed in 50 cc. centrifuge tubes, and slanted until coagulation has occurred. After standing for a few hours at room temperature, the clots are broken, and the tubes of blood stored overnight at 4° C. The next morning the serum is drawn off, centrifuged, pooled and preserved with 0.3 per cent (final concentration) of tricresol.

d. Testing of serum. The pooled serum of each type is tested for type-specific Neufeld reactions and for heterologous reactions with known closely related types. For these tests, mice are injected intraperitoneally with cultures of the type of pneumococcus desired and the peritoneal exudate washed out with saline solution about six hours after inoculation. Neufeld tests are set up in the usual manner, and if satisfactory results are obtained and no cross-reactions observed, the serum may be either filled immediately into separate containers or added to the stock supply of serum of the respective types.

e. Dilution of serum. It is possible, after high titered serums are obtained to obtain satisfactory Neufeld reactions with type-specific serums diluted with normal rabbit serum. The degree of dilution must be determined by tests, taking into consideration the amount of dilution which may occur in making the pools used in routine type determination in the diagnostic laboratory.

ANTIPNEUMOCOCCIC RABBIT SERUM FOR THERAPEUTIC PURPOSES

● Horsfall and Associates (*Science 84*, 579 (1936)) reveal the different properties of antipneumococcus horse serum and antipneumococcus rabbit serum. The sera from the two species have been found to differ in at least 30 respects in their immunological properties. Many of the differences indicate that the product from the rabbit possesses certain advantages over the horse serum preparation. Rabbits respond to pneumococcus immunization with the production of serum of high titer so that concentration is less necessary as compared with products from horse serum. Anaphylactic reactions are infrequent, of a mild character, and individuals generally are not sensitized to rabbit serum as they are to horse serum. It appears that the antibodies in immune rabbit serum are of smaller size than are those in horse serum, so that diffusion of the immune substance into infected foci may occur with greater readiness than in the case of horse serum. If specific serum treatment is to be employed in all cases due to any of the 30 serological types, the production of these serums will be greatly simplified and the cost will be less if small animals as rabbits are used rather than horses. Clinical trials with antipneumococcic rabbit serum in the treatment of pneumonia are encouraging (*J.A.M.A., 10,* 1483 (1937); *113,* 1314 (1939)). Rabbits are

immunized more quickly and the cost of producing such serum is much less. The following method of production, processing and standardization as employed at the Rockefeller Institute for Medical Research is given by Goodner and Associates, (*J. Immunol.*, *33*, 279, (1937)).

Suspensions of Pneumococci For Immunization

Cultures. Cultures of pneumococci of the different specific types are used in the smooth phase; whenever possible mouse-virulent strains are selected. The cultures are maintained in rabbit blood broth at 37°C.; the serial transfers are made at two day intervals using a loopful of cultures as inoculum. Under these conditions most strains remain in the smooth phase and retain their mouse virulence for long periods of time.

Immunizing Suspensions. Suspensions of killed encapsulated pneumococci used for immunizing purposes have been prepared by the following three methods.

a. Heat-killed suspensions. The pneumococci are grown for 18 hours in beef-heart infusion broth. At the end of this time the organisms are collected by centrifugation and resuspended in an amount of saline equal to one-tenth the volume of the original culture. This suspension is then immediately heated at 70° C. for 30 minutes.

b. Formalinized suspensions. The organisms are similarly grown for 18 hours and at the end of this time formalin is added to a final concentration of 1%. The formalinized culture is stored in the ice box for 24 hours, then centrifugalized and the cells resuspended in one-tenth the original volume of 0.1% formalinized saline.

c. Heated formalinized suspensions. Suspensions are prepared according to the method with formalin and are then finally heated at 80° C. for 30 minutes.

Route of Immunization. The intravenous method of immunization has greater advantages in the production of immune serum of high titer.

Rabbits

Breeds. After experience with a large number of breeds of rabbits certain preferences have developed regarding animals which are easily handled and which appear to be readily immunized. The New Zealand Red and the Chinchilla are among the better breeds for this purpose since both have good ears and are not too nervous.

Weights. Weight is apparently an important consideration only in the case of immunization with type III pneumococcus, a notoriously poor antigen. The most suitable weight for immunization with this type of pneumococcus is in the range of 1600 to 2800 grams. Weight may also play some part as regards other types of pneumococcus but it is not sufficiently important to overbalance the other advantages to be gained from the use of larger animals.

Care and housing. The rabbits in this laboratory are kept in individual cages and protected from draught as completely as possible. The ration consists of hay, oats, and cabbage. No water is given.

Immunization and Bleeding Schedules

Schedules have been worked out to fulfill two purposes, viz., prompt immunization and maintenance of the productive life of the animal over the longest period of time.

Initial immunization. The method routinely employed is termed the "four-week complete" system. The rabbits receive three courses of four intravenous injections, the injections in each course being given at daily intervals and the amounts adjusted to give increasing dosage in each course. During the first three weeks each animal receives the equivalent of 63.5 cc. of culture.

It is possible by several other plans of immunization to obtain initial titers somewhat higher than those produced by the above system but the immunization must not be too intensive if it is desired to maintain a reasonable titer over a long period of time. It not infrequently happens that an animal rapidly forced to produce antiserum of very high titer will subsequently produce only serum of low titer.

Bleeding schedule. The rabbits are first bled 11 days after the last injection of the third course. Thereafter bleedings are carried out every two weeks. A recuperation period must be provided at suitable intervals. In this laboratory every sixth scheduled bleeding has usually been omitted.

Maintenance injection schedule. Each rabbit receives an additional course of injections immediately following each bleeding, that is, one immunizing course every two weeks. These are continued even when the animal is in a recuperation period in so far as the bleeding schedule is concerned.

Bleeding. Rabbits are bled from the heart. Blood is removed from the heart slowly, care being taken to avoid too much negative pressure. Fifty cubic centimeters of blood can routinely be taken from animals weighing 1500 to 2500 grams and 60 cc. from larger animals.

The blood is placed in 250-cc. sterile centrifuge bottles and allowed to clot. The clot is rimmed and after the bottles have stood overnight in the ice box the serum is drawn off and filtered through a Berkefeld V. candle. Merthiolate is added to a final concentration of 1:10,000.

Processing of Serum

Immune rabbit serum in the raw state is almost invariably toxic for man. In order to reduce this undesirable effect the serum must be treated before it is used for therapeutic purposes. The processing is carried out as follows:

The serum is first heated at 56° C. for 30 minutes. This heating process is so very important as regards the reduction in toxicity that the greatest care must be exercised to bring out every part of the fluid to the proper temperature. This should not exceed 58.5°C. at any point nor must the temperature of any other part of the fluid be below 56° during the time period.

For the process of adsorption it is necessary to prepare sterile washed kaolin in advance. Dry commercial kaolin (in the proportion of 25 grams for each liter of serum to be treated) is washed with physiological saline by centrifugation. The moist kaolin cake is then sterilized in the autoclave.

The serum which has been already heated to 56°C. and subsequently cooled is added to the sterile washed kaolin, and the kaolin is worked up into the serum very thoroughly. The

mixture is allowed to stand overnight in the ice box and the serum is then separated from the kaolin by centrifugation. The serum is again filtered through a Berkefeld V. candle and introduced into the final containers.

After the serum vials have been capped they are immersed almost up to the neck in water and heated at 56°C. for 30 minutes.

Each of these steps has been found to be important and essential and none should be omitted if the serum is to be used for therapeutic purposes.

It may be concluded from these and other tests that the processing of antipneumococcic rabbit serum as described caused no significant loss of specific antibody.

Test For Toxicity of Serum Preparations

Immune rabbit serum which has not undergone the processing just described is almost invariably toxic for man. Characteristic chills and febrile reactions follow the use of the untreated rabbit antiserum. With the introduction of the various steps in processing this pyrogenic effect is greatly reduced but it has been found that a few lots of serum even though treated still produce chills in patients.

Routine Sterility Tests

Tests as to sterility are carried out as prescribed by the National Institute of Health, Memorandum of December 19, 1934. Since all the procedures of bleeding and processing are completely aseptic in every step contaminated sera are not encountered.

Standardization of Antipneumococcic Rabbit Sera

Since it appears possible to produce antipneumococcic rabbit serum for therapeutic purposes against all types of pneumococci, the problem of standardization becomes important. As routinely practiced for antipneumococcic horse serum, various sera have been selected or are being selected as arbitrary standards for each type. This necessitates the use of mouse protection tests for the standardization of any lot of serum, a requirement which involves considerable expense and labor. Mouse protection tests can be carried out with a measure of precision only when a large number of mice are used for each serum. Moreover certain strains of pneumococci are relatively avirulent for mice and are therefore not suitable for protection tests. Perhaps the least desirable feature to this entire system is the matter of arbitary standards of unitage potency for each type serum. In other words no matter how carfully the standard sera are chosen the values as referred from one type to another will of necessity remain at variance.

Fortunately for purposes of standardization of antipneumococcic rabbit serum there exists at least with type I an excellent correlation between the maximum amount of specifically precipitable protein and the mouse protection potency. A method of determining the specifically precipitable protein would therefore serve as an excellent method for standardization were it not for the fact that different preparations of pneumococcic capsular polysaccharide vary greatly in their capacity to precipitate the type-specific antibodies of antipneumococcic rabbit serum depending upon the method employed for the isolation of the carbohydrates. Furthermore, the use of the quantitative determination of precipitin would require the isolation of the capsular polysaccharides of many different types of pneumococci, which is in itself a laborious and expensive process. It seemed necessary therefore to find some other precise method in order to simplify the quantitative determinations of the antibody to the greatest possible extent.

The antipneumococcic rabbit serum to be tested is diluted exactly 1:8 with 0.85 per cent saline. Two cubic centimeters of the diluted serum, containing 0.25 cc. of the original serum, is then accurately pipetted into each of four thick walled centrifuge tubes. To each of two of these tubes is then added exactly 1.0 cc. of a suspension of heat-killed homologous pneumococci, prepared as previously described. Into each of two additional centrifuge tubes exactly 1.0 cc. of the same suspension of pneumococci is pipetted. The contents of each of the six tubes are then made up to exactly 5.0 cc. by the addition from a calibrated burette of an adequate amount of 0.85 per cent saline. The tubes are capped, and their contents twirled vigorously so that a thorough mixing is rapidly effected and they are then incubated for two hours at 37° C. After this time the contents of the tubes are again thoroughly mixed by vigorous twirling, and the tubes are placed in the icebox for 18 hours. The contents are then vigorously twirled the third time and the tubes are spun at 2500 RPM for one hour at 4° C. in the angle centrifuge.

This packs the bacteria, both agglutinated and non-agglutinated firmly in the tips of the tubes and leaves perfectly clear supernatant solutions. 2.0 cc. aliquots are then pipetted from each supernatant solution. These are digested and analyzed for total nitrogen (J. Exp. Med. 64, 583 (1936)). The mean of the duplicate determinations on the supernates of the tubes containing only bacterial suspensions is subtracted from the mean of the duplicate determinations on the supernates of the tubes containing both antiserum and vaccine. The figure thus derived is then subtracted from the mean of duplicate determinations on the supernates of the tubes containing only serum. This figure when multiplied by ten is equivalent to the agglutinin-nitrogen per cc. of the serum under these conditions, since the total nitrogen was determined on 2 cc. of a total volume of 5.0 cc. containing 0.25 cc. of the original serum.

With type I antipneumococcic rabbit serum it has been determined that on the average 1 mgm. of specific agglutinin-nitrogen, or 6.25 mgm. of specific agglutinin-protein, is equivalent to 1100 mouse-protective units, in comparison with standard sera. Since the relationship between protective potency and the content of specific antibody of antipneumococcic rabbit serum appears to be constant, it is suggested that for rabbit sera this relationship be used for all types.

The general system for unitage evaluation as it applies to antipneumococci rabbit sera is unnecessarily laborious and under the usual conditions rather inaccurate, whereas quantitative estimation of specific antibody is both simple and precise. Since the unitage system is originally adopted in the absence of precise methods of potency estimation and since more often than not a unitage value represents an approximation rather than an accurately de-

termined potency it would be more exact to evaluate rabbit antisera on the basis of actual antibody content. If the method suggested proves valid there seems to be no reason why antipneumococci rabbit sera could not be assayed in some such way as that described as to the precise content of specific antibody-protein and the containers so marked. With the present knowledge as to the properties of antipneumococcic antibodies of the rabbit it seems as rational to use a definite number of milligrams of antibody therapeutically as it is to use quantitative amounts of any therapeutic agent. Furthermore, the adoption of such a system would permit of the treatment of patients with accurately determined amounts of antibody and in due time it might be expected that quite an exact basis for adequate antibody dosage in lobar pneumonia could be arrived at.

OTHER CONSIDERATIONS OF ANTIPNEUMOCOCCIC RABBIT SERA

● Many procedures other than given have been presented for the production of antipneumococcic rabbit serum. Among them are to be found the method described in detail by Fritiz (*Amer. J. Publ. Health, 29,* 224 (1939)), and a thorough description of the study of cross reactions in the production and standardization of diagnostic sera (*Amer. J. Hyg., 29,* 103 (1939)). Type I pneumococci inactivated by the photodynamic action of methylene blue instead of by heat or formalin give rise on intracutaneous injection into rabbits of a highly potent antiserum (*Proc. Soc. Exptl. Biol. Med., 30,* 159 (1938)).

USES

● Antipneumococcus Serum or the concentrated products prepared from the latter are mainly antibacterial and only slightly antitoxic. The curative and protective value of these pneumococcic preparations depend upon specific bacteriolysins, bacteriotropins, and antitoxic substances especially the neutralization of a specific toxic material which may be the agent responsible for fever-production. In practice today the refined and concentrated Anti-Pneumococcic Sera (from horses and preferably rabbits) or Pneumococcus Antibody Globulin prepared by Felton's method of concentration are mainly used (*J.A.M.A.,* 1928, p. 1503, 2035). The use of most of the other products has been discontinued. Concentrated and refined pneumonia sera possess the advantage of small bulk, more rapid absorption, elimination of a large amount of inert serum protein and lipoids as well as chill-producing factors, and produce a quicker therapeutic response. They are less provocative of serum sickness as these products contain less than 10 per cent of the proteins present in unconcentrated serum. It is thus possible to employ intramuscular injections of such concentrated products wherever intravenous administration is not possible. The dosage intramuscularly is twice the intravenous dose. A disadvantage of Felton's serum, however, is the presence of a fever-producing substance so that thermal reactions may appear one-half to one and a half hours after the administration. Where serum sensitiveness is suspected, proceed as directed under Diphtheria Antitoxin for sensitiveness to horse serum and desensitization or see Lord and Heffron's "*Lobar Pneumonia and Serum Therapy*" (*Commonwealth Fund,* 1936).

ADMINISTRATION

● The antipneumococcic serum or serum product for intravenous administration should be warmed to body temperature (avoid heating above 98°F.) and it is injected slowly intravenously, allowing at least 10 to 15 minutes for the injection, the first portion being allowed to pass in more slowly than the later portion. It may be diluted with 3 to 5 volumes of sterile normal saline solution. Intravenous injections of 10,000 or 20,000 units are given every two hours until 100,000 or more units have been administered within

the first 24 hours or until a beneficial effect has been obtained. Subsequent doses are given on the second day and the intervals between injections can be extended to twelve hours if treatment was started during the first three days (*J.A.M.A.*, *95*, 1547 (1930); *98*, 779 (1932)). If two lobes are involved, the total dose is increased to at least 150,000 units. The presence of a bacteremia is an indication to double the usual total doses.

Belk and Associates (*Am. J. Med. Sci.*, *187*, 844, (1934)) recommend the use of Pneumococcus Antibody Solution in pneumococcus lobar pneumonia regardless of the type of pneumococcus. A report of the Therapeutic Trials Committee of the Medical Research Council of Great Britain on the serum treatment of lobar pneumonia is of interest (*Brit. Med. J.*, Feb. 10, 1934). Antipneumococcic Serum, type VII has been recently marketed after Cooper and Associates (*J. Exp. Med.*, *55*, 531 (1932)) in classifying group IV pneumococcus into 29 types, identified group VII and VIII in many of their cases. Bullowa (*J.A.M.A.*, 1934, *102*:1560) reports it was found possible to produce a type VIII serum of relatively high potency as estimated by the Felton unit test. Finland and Coworkers (*Am. J. Med. Sci.*, *193*, 43 (1937)), present interesting and valuable results obtained with type VII antiserum and Benjamin and Coworkers (*Ann. Internat. Med.*, *XI*, 437, (1937)) reveal satisfactory results in specific serum therapy against types I, V, VII, VIII, XIV and XVIII. Cecil (*J.A.M.A.*, *108*, 689 (1937)) shows the marked value of serum therapy.

DOSAGE

● Antipneumococcic serum is also used in pneumococcus meningitis and pneumococcus peritonitis. Children under 12 years of age stand pneumonia quite well. The death rate is rather low. These reasons and the fact that (horse) serum therapy sensitizes the child to horse serum, thus making subsequent administration of diphtheria and other antitoxins somewhat hazardous, have resulted in the fact that serum therapy has not assumed an important role in the treatment of pneumonia in children. The marketing of rabbit serum products may influence the more frequent use of (rabbit) serum therapy in pneumonia among children. Cole (*J.A.M.A.*, *109*, 2059, (1937)) emphasizes the need of large doses as early as possible; larger doses for type II than type I cases; and in types V, VI, VII and VIII pneumonias, inasmuch as the disease is not as severe, smaller doses of specific serum are given. In his report of 500 cases of type I pneumonia treated with specific serum, the mortality was under 4.8% for patients treated in the first 3 days, 8% for those treated on the 4th day, 8.6% on the 5th day and 19.5% on the 6th day. The same worker reports only few severe anaphylactic reactions, all of which were promptly controlled by epinephrine. Other workers advocate preliminary skin tests as in all testing and therapy with serum products. Contraindications to serum therapy are for patients in a state of shock, or those possessing pulmonary edema or severe cardiac disease. Serum therapy should be employed with caution for sufferers from hay-fever, asthma, antineurotic edema or those sensitive to horse serum.

As mentioned previously, the factors influencing dosage are: the type of pneumococcus causing the infection; age of the patient; presence or absence of a bacteremia; extent of pulmonary involvement; duration of the disease; presence of complicating conditions as pregnancy, etc.; how soon after illness serum therapy was employed; etc. Infections caused by type I, IV and V respond more readily to serum therapy than do those caused by types

II, VII, or VIII, with the result that more serum may be required to control the latter type cases. A larger dosage is required for type II than for the other types. Patients under 30 years of age should receive within 12 to 24 hours the following minimum dosage for each of the respective types: 60,000 units for type I; 100,000 units for type II; 200,000 units for type III. The treatment of types IV and V patients is as per schedule given for type I. Types VII and VIII patients are to receive dosage as given for type II. In patients over 30, or with two or more lobe involvements, or with positive blood cultures, the units should be doubled. Treatment should be continued until beneficial clinical improvement is evident. Type XIV serum seems to be effective, but the dosage cannot be given in units as above since this type of pneumococci is not mouse virulent. Finland (*New England J. Med.*, *220*, 336, (1939)) reports on the favorable results of specific serum treatment in infections by pneumococci of the "higher" types.

VALUE

● It has been difficult to determine definitely the value of serum therapy in pneumococcus infections and even in lobar pneumonia due to many factors. No single agent or means is known today which can be employed to treat successfully every case of lobar pneumonia. Certain definite means are, however available, the judicious use of which will result in a reduction of the mortality rate. The great majority of cases of lobar pneumonia, a considerable percentage of cases of bronchopneumonia, and an undetermined percentage of cases of postoperative pneumonia are caused by pneumococci. Every reasonable effort should be made to determine the etiologic organism in every case of pneumonia at the earliest possible moment. Bacteriologic study of the sputum need not await Roentgenologic confirmation of the diagnosis. In urgent cases a bacteriologic diagnosis can be made within an hour or less. It is absolutely necessary that the type of pneumococcus be determined because serum is of no significant value except against its homologous type of organism. The specimen submitted for bacteriologic examination must represent exudate expectorated from the lung and it should be examined promptly. It is necessary to instruct both the patient and nurse clearly about the importance of submitting a suitable specimen for examination. Sometimes, it is necessary to examine more than a single specimen. Blood should be obtained for culture in every case of lobar pneumonia and sometimes this procedure should be repeated. Therapeutic approach is greatly modified and the prognosis gravely affected by the presence of bacteremia. Serum is the only effective treatment for bacteremia and the mortality rate continues high in spite of such treatment.

AVOID DELAY

● The recently marketed types of rabbit serum are administered somewhat differently from horse serum. Rabbit serum is prone to produce an early febrile reaction, but usually this is not serious except among patients who have an unusually high fever. It is possible to administer rabbit serum more rapidly, in larger amounts, and in fewer doses than horse serum. The effectiveness of serum therapy is multiplied by early administration and many of the apparent failures are directly attributable to late treatment. For this reason, it is rarely permissible to withhold serum because of mild initial symptoms. There is no dependable method of forecasting the eventful severity of the disease on the onset. The error of delay may permit extension of the disease to other lobes and possibly bacteremia or complications

with greatly increased cost and hazard. When serum therapy is delayed until the fourth day or later, the amount of serum required is much greater and in an effort to conserve the patient's financial resources he may be subjected to increased expense. The apparent high cost of serum is mentioned constantly as a reason for withholding it from patients. When the actual value of serum is realized this objection vanishes. At present prices, adequate early serum therapy costs no more than two or three days of oxygen therapy with continuous private nursing service. Actually it is possible for serum therapy to save expense by shortening the period of hospitalization, by decreasing costs of oxygen therapy and special nursing, and by preventing extremely expensive complications. The effectiveness of modern serum therapy in cases of pneumonia no longer can be a subject for debate. When adequate doses are administered on the first or second day of the crisis, it will lead frequently to prompt termination of the disease by crisis. It has definitely been proved that the mortality associated with pneumococcus pneumonia may be halved by serum therapy if specific-type serum is used early, in adequate dosage and persistently.

Finland and Brown (*Amer. J. Med. Sci., 197,* 151 (1939)) report on the effectiveness of type I pneumococcus horse and rabbit antisera during a period of about 10 years. The mortality was 19% among serum-treated and 40% among the non-serum-treated cases of pneumonia. Blankenhorn (*J.A.M.A., 111,* 1260 (1938)) states that refined and concentrated horse serum for types I and II pneumonia is a well established and satisfactory remedy, reducing within 3 years the mortality from pneumonia 76%. Bullowa (*Penna. Med. J., 42,* 17 (1938)) gives the following data: In type I pneumococcus pneumonia, the death rate has been reduced from 30% in untreated cases to 8.5% in serum-treated cases; in type II infections the difference in mortality of treated and untreated cases is 10%; and in type XIV the death rate has been reduced by serum treatment from 33% to 17.6%. Horn advises the use of type specific serum in every case of pneumonia where serum is available (*Ann. Internat. Med., 12,* 922 (1939)).

OTHER PNEUMOCOCCUS PRODUCTS (POLYSACCHARIDES)

● The pneumococcus is an encapsulated bacterium. In this capsule of the pneumococcus there have been found complex polysaccharides. The chief polysaccharide of each type of pneumococcus is chemically distinct. Considerable data have been reported concerning these polysaccharides (*J. Exp. Med., 64,* 843, (1936); *Bull. Johns Hopkins Hosp., 59,* 114, (1936)). Cecil (*J.A.M.A., 108,* 2156 (1937)) reports on the use of a specific polysaccharide substance to detect whether adequate amounts of serum have been used in treatment and Tilghman and Finland (*J. Immunol., 31,* 239 (1936)) present a study of the cutaneous reactions to type-specific polysaccharides following serum injection. Several experimental reports have been presented on specific capsular pneumococcus polysaccharides and the immunity induced, but David (*J. Immunol., 33,* 1, (1937)) in considering the response of 15 infants from 2 to 15 months of age to inoculation with type I pneumococcus carbohydrate reveals that only one responded with demonstrable humoral type I pneumococcus antibody. More recently Finland and Associates (*J. Clin. Investig., 17,* 479 (1938)) showed that type-specific polysaccharides prepared from pneumococci of types I, IV, V, VII and XIV were capable of stimulating the production of homologous type-specific antibodies in human subjects when administered subcutaneously or intracutaneously. An inter-

esting report was presented on the lower incidence of pneumonia in 61,000 civilians inoculated with an antigen containing equal parts of types I and III pneumococcus polysaccharides as compared with control groups (*U. S. Publ. Health. Rep.*, *53*,, 1877 (1938)). (For further details see *"The Biology of Pneumococcus"*, White and associates, *Commonwealth Fund,* 1938) and *"Pneumonia"*, R. Heffron, Commonwealth Fund, 1939).

PNEUMOCOCCUS VACCINE

● The use of pneumococcus vaccine for prophylactic immunization against pneumonia and even in the treatment of the latter has received some attention (*Ill. Med. Jour.*, 1928, III, 280). Doses of from 5,000,000 to 50,000,000 attentuated bacteria and partially autolized preparations from pneumococci have been used with indications of some apparent degree of successful influence on the clinical course of some cases of lobar pneumonia (*J. Exp. Med.*, 1931, *53*:567). The benefits appear to be due to non-specific elements. Saline suspensions of attenuated bacteria in three to four doses, given subcutaneously at intervals of five to seven days have been used for prophylaxis with some success. Oral administration has been tried. The drawback against mass or universal active immunization is the relatively short immunity which results (about six months) and the fact that immunization against all types of pneumococci during one series of treatments is not possible.

Mixed vaccines consisting of several predominating types of pneumococci and strains of streptococci, Friedlander bacilli, *Staph. aureus, M. catarrhalis,* and *H. influenzae* have been used effectively to reduce the incidence of pneumonia and other respiratory infections (*Lancet, 1,* 87 (1914); *Pub. So. Afr. Inst. Med. Res.*, *7,* 1, 83 (1935)). Felton has prepared a pneumococcus vaccine which appears to reduce the incidence of pneumonia by half and apparently can be used to demonstrate (by skin testing) susceptibility to pneumonia (*Science News Letter, 35,* 278 (1939)).

COMBINED TREATMENT

● A large number of reports reveals the value in pneumococcus pneumonia of the newer chemotherapeutic agents, sulfapyridine in all types and sulfanilamide in type III cases. A review of the literature, however, reveals that the combined therapy of specific antisera with these drugs is more valuable and is to be advocated as the treatment of choice. Hydroxyethylapocupreine has recently been described as equal in effectiveness in the treatment of pneumonia but without the toxic effects of sulfapyridine and sulfanilamide.

XI

OTHER ANTIMICROBIC SERA

SERUM ANTIDYSENTERICUM

Antidysenteric Serum; Serum Antidysentericum, Polyvalent; Serum Antidysentericum (Shiga) (B. P.)

DYSENTERY, either as severe or mild inflammations of the lower intestinal tract characterized by frequent and painful evacuations of fluid or mucopurulent bloody fecal discharges has afflicted man for many centuries. The inciting cause of these disorders grouped under the clinical term dysentery may be either amebae (*E. histolytica* producing amebic dysentery) or a group of closely allied bacilli (producing bacillary dysentery). The former is most frequently the cause of cases of chronic dysentery. In temperate climates acute dysentery is rarely caused by amebae but usually by the bacilli of dysentery. It was soon recognized that a number of bacterial species comprise the latter. At present *Shigella (B.) dysenteriae* (Shiga bacillus) and *Shigella paradysenteriae* (comprising the Flexner, Hiss (Park-Hiss-Russell), Strong and Sonne varieties of the dysentery bacillus) are accepted as the causative agents in clinical dysentery (*"A System of Bacteriology"*, 1929, London, IV, 161, 220). The distinct types of dysentery bacilli vary greatly in their powers to produce exotoxins, the strongest soluble toxic products being produced by the Shiga variety and less regularly and only mild toxins are produced with bacilli of the paradysentery type. In fact the Shiga variety of the dysentery bacillus is regarded as producing a true exotoxin (*J. Exp. Med.*, 1920, xxxi, 19) and the disease caused by it is probably a true toxemia. The early recognition of the infective agent in a case of dysentery furnishes the key to the success in treatment. Therapeutic measures for the amebic dysentery are different than for bacillary dysentery and serum therapy is of value only in the treatment of the latter and especially if specific antibacterial serums are employed.

ANTIDYSENTERY SERUM

● Antidysentery serum consists of the native blood-serum of animals (usually horses) which have been immunized against the *Shigella dysenteriae* (Shiga bacillus) and its soluble products of growth (producing a monovalent serum) or against this and other types of the dysentery bacillus producing a polyvalent serum. Usually the strains selected are those representative of the principal types present in the country where the

preparation is to be used. In the United States, in the preparation of a polyvalent antidysenteric serum, immunization of the animals is carried out against several strains of Shiga and Flexner or these with Hiss-Y types of dysentery bacilli. The antidysentery serums prepared with the Shiga strains (official in the British Pharmacopoeia) are not only antibacterial but also antitoxic as this type yields a true extracellular toxin which the other strains of dysentery bacilli do not produce. The technique employed in preparing antidysentery serum is the same as that given under Antimeningococcus Serum. Flexner and Amoss (*J. Exp. Med.*, *21*, 515 (1915)) prepared a polyvalent serum by injecting intravenously heat-killed, followed by living organisms.

MARKETABLE ANTIDYSENTERY PRODUCTS

● The whole serum, either liquid or dried or the pseudoglobulins to which are attached the specific antitoxic bodies (these being obtained as mentioned under the other antitoxins), either in solution or dried, may be employed and are marketed. Sterility and toxicity tests are performed as on other biological preparations. If dried, an antiseptic is not added, but a suitable antiseptic may be added to the liquid preparations. The marketable antidysentery serums or other products (all forms) are distributed in sterile glass containers, properly labelled with the potency recorded in terms of units and the expiration date is to be given. The liquid serum is yellow or yellowish-brown and when dried or as dried globulins the product appears as a yellow, yellowish-white or yellowish-brown powder or in flakes. The solution of globulins is of a yellowish-brown or yellowish-green color. The liquid forms are odorless except for the odor of added antiseptic and are usually transparent when first prepared but may become opalescent during storage. This preparation (all forms) is standardized in Great Britain by mouse protection tests, by comparing the quantity of marketable serum required to protect mice against the lethal effect of dysentery (*Shigella dysenteriae*) toxin with the dose of a standard antidysentery serum (Shiga) necessary to give the same protection. The standard preparation and the unit thereof are given in the Regulations made under the Therapeutic Substances Act of 1925 and the former is supplied by the National Institute for Medical Research in London. The polyvalent antidysentery serum most frequently used in the United States is standardized by determining its agglutinative titer against standard strains of the types of dysentery bacilli used for immunization and especially determining its antitoxic potency when combined with a previously standardized toxic filtrate of the Shiga dysentery bacillus. Control standard serum and toxin are obtainable from the National Institute of Health. Attempts have been made to separate the globulins containing the specific antibodies by fractional precipitation and to prepare a refined and concentrated antidysentery serum.

O'Brien and Coworkers (*Brit. J. Exptl. Path.*, *5*, 100 (1924); *6*, 84 (1925)) present details in the preparation of a dried test powder employed for standardizing Shiga bacillus antiserum and an improvement of this method is given by Blake and Okell (*J. Path. and Bact.*, *32*, 121 (1929)). A polysaccharide precipitative test is described as being of value for the standardization of antibacterial dysentery serum (*Brit. J. Exptl. Path.*, *13*, 28 (1932)), but it is not suitable for estimating the antitoxin content (*J. Immunol.*, *29*, 435 (1935)).

VALUE AND DOSAGE

● Antidysentery serum prepared for the Shiga type of dysentery bacillus (which is found most frequently in Europe and Asia) has yielded fairly satisfactory results as a valuable therapeutic agent against this type of bacillary dysentery (*Lancet,* 1924, ii, 232). Antiserums for the paradysentery strains (most frequently found responsible for the dysentery in the United States) have not been found to be as efficient in the treatment of dysentery caused by these varieties (*J.A.M.A.*, 1921, *77*:1863). Since the efficacious Shiga bacillus antiserum is specific, this monovalent preparation is employed mainly in European and Asiatic countries, where infections with this type prevail. Antiserums for the other types of dysentery bacilli have not proven of as much value and are not so favorably received. As a prophylactic dose, 10 cc. may be given subcutaneously. The therapeutic dose given as early as possible is from 50 to 100 cc., administered intramuscularly or preferably intravenously, to be followed at 8 hour intervals by doses of 50 cc. each until normal symptoms prevail (*Lancet,* Aug. 2, 1924, p. 232). Lantin (*Am. J. Med. Sci.*, 1930, *180*:635) states that the serum is incapable of dislodging toxin which has become anchored to the tissue cells and points out that the value of serum therapy is enhanced if given early in the disease. He also employed the rectal method of administration with encouraging results. Bieling and Meyer (*"Heilsera und Impstoffe in der Praxis,"* 1932, Leipzig) present convincing evidence of the value of serum therapy in the treatment of dysentery.

VACCINE THERAPY IN BACILLARY DYSENTERY

● Vaccine therapy has been employed especially in the chronic forms of dysentery and even in acute forms which show little tendency towards cure (*J.A.M.A.*, 1919, *73*:1177). Oral administration has also been practiced (*Deut. Med. Wochnschr.*, 1925, *51*:1728). For further details see "A *System of Bacteriology,*" London, 1929, iv, 212, 240.

Plain bacterial vaccines (*Wschr. Kinderhlk.*, 1926, xxxiii, I) and formolized vaccines (Anacultures) (*Jr. Infect. Dis.*, 1925, *37*:125) have been used as prophylactic agents against bacillary dysentery. Oral administration has also been used (*Ann. Inst. Past.*, 1922, *36*:579). Results are given on the use of the oral administration of 3 pills at 3 day intervals, each pill containing 30 mg. of dried bacilli (*Bull. Off. Int. Hyg. Publ.*, *25*, 639 (1933)). Formolized dysentery toxoids (*Ann. Inst. Past.*, *40*, 134 (1926)) and serovaccines (*J. Roy. Army Med. Corps.*, *28*, 615 (1917); *30*, 476 (1918)) have been advocated. It appears that prophylactic immunization against bacillary dysentery has not proven of value for wide use and at present its employment is not to be advocated on a large scale as a general protective measure.

ANTI-ANTHRAX SERUM

ANTI-ANTHRAX Serum (Serum Antianthracicum) is prepared by immunizing horses against virulent anthrax bacilli (*'A System of Bacteriology'*, 1930, London: H. M. Stat. Office, V. 439). Cattle have been, and are, injected intravenously with living organisms to prepare a marketable bovine anti-anthrax serum. For effective results, these sera are to be administered at the earliest possible moment in large doses. In slight cases,

intramuscular injections are given. In severe cases, initial doses of from 100 to 200 cc. are administered intravenously. Injections of 20 cc. to 50 cc. each are repeated every 6 to 12 hours, or at suitable intervals if indicated, or until blood cultures are negative. Eichorn (*J. Am. Vet. M. Assoc.*, 1925, *68*:276) has developed a spore vaccine from carefully tested strains of *B. anthracis*, and has prepared and concentrated an anti-anthrax serum, which is employed not only in lower animals but it has been used increasingly in the treatment of human anthrax (*Month. Bull. N. Y. City Dep't. Health*, 1923-23, *14*:3 and 7). Stamtin (*Ann. Inst. Pasteur, 61*, 394 (1938)) advocates the immunization of laboratory animals and of sheep against anthrax by the use of strains of *B. anthracis* which have lost the power to form capsules but which are capable of producing edema. The 'barrage' of antianthrax serum injected locally around the lesion as suggested by some workers has been replaced by intramuscular and intravenous injections (*Centralbl. f. Bakteriol.*, I, Abt., 1926, *99*:1242). Reports indicating that arsphenamin or neoarsphenamin is useful as a chemotherapeutic agent have resulted in the simultaneous administration of anti-anthrax serum to which is added 0.9 Gm. or less of neoarsphenamin dissolved in 100 cc. of sterile saline solution (*Ztschr. f. Hyg. u. Infectionskrankh.*, 1926, *105*:509).

From available data, it appears that the serum treatment of anthrax in man has resulted in lowering the mortality (*Lancet, 2*, 594 (1928); *Brit. Med. J., 2*, 50 (1933)) by two-thirds or from 25 percent to less than 8 percent. Surgical excision or incision and drainage of the local lesion is not generally advocated in conjunction with serum therapy. For further details see *Industrial Anthrax Report* (*Suppl. to Am. J. Pub. Health, 25*, No. 2 (1935)).

ANTISTREPTOCOCCIC SERUMS

STREPTOCOCCUS infections are of relatively frequent occurrence. Their acute and serious character has drawn the interest of many workers who have attempted to develop an efficient serum therapy. Many different immune streptococcus serums have been developed and marketed, these being prepared according to different methods by using various cultures of toxins or both. *Streptococcus Scarlet Fever Antitoxin* and *Erysipelas Streptococcus Antitoxin* are streptococcic curative serums considered previously.

POLYVALENT ANTISTREPTOCOCCUS SERUMS

SERUM Antistreptococcicum, marketed as *Antistreptococcic* or *Antistreptococcus Serum* (*Polyvalent*), is the blood serum of horses (or other animals) which have been immunized against strains of streptococci representing each of the well known pathogenic varieties. A serum which is antitoxic as well as antibacterial may be prepared by immunizing the animals against the soluble toxins as well. Such a product is dependent for its action upon bacteriotropins, antitoxins and possibly bacteriolysins. A refined and concentrated serum consisting of the globulins to which are attached the antibacterial and antitoxic substances from an immune serum

is also marketed. Polyvalent Antistreptococcic Serum is employed in the treatment of streptococcal infections (*J. A. M. A.*, 1926, *86*:189; 1929, *93*:1524; 1934, vol. *103*, 2062) as in septicemia, pyemia, sapremia, wound infections, puerperal sepsis, endocarditis, alveolar abscess, periostitis and osteomyelitis. The action of this serum is erratic and at times even unfavorable (Kolmer: 1924, *Infection, Immunity and Biologic Therapy*); (*J. A. M. A.*, 1926, *86*:189). It has thus become difficult to determine the exact efficacy of this curative serum (*Lancet*, *I*, 761, 815, 867). On this account there has been the tendency to prepare specific streptococcic antiserums, the latter made by immunizing animals against specific strains of streptococci; and the antiserum is to be used only in the treatment of diseases caused by such specific types of streptococci. Accordingly the following antistreptococcic serums are also marketed:

HEMOLYTIC ANTISTREPTOCOCCUS SERUMS

Antistreptococcic Serum Hemolytic is prepared by immunizing horses against various strains of hemolytic streptococci and contains antibodies specific for the latter. *Antistreptococcus Serum Encephalitis Lethargica*, prepared by immunizing horses against the soluble toxin from, as well as against the organism itself, a strain of streptococcus isolated from a case of epidemic encephalitis. *Antistreptococcus Serum, Poliomyelitis* (Antibody Globulin), (*J. A. M. A.*, *77*:588, 1921; *94*:777, (1930)) prepared by immunizing horses against several strains of streptococci obtained from cases of poliomyelitis. *Streptococcus Cardioarthritidis* (*S-C-A Serum*), prepared from the *Streptococcus cardioarthritidis* associated with chorea and acute rheumatic fever and recommended in the treatment of the acute forms of these conditions when the diagnosis has been clearly established (*Am. Jour. Med. Sci.*, *173*:101, (1927); *175*:638, 650, (1928)). *Ulcerative Colitis Antistreptococcic Serum* (refined and concentrated) and *Streptococcus Antibody Globulin* (Bargen Serum) prepared from live cultures of a nonhemolytic streptococcus (diplostreptococcus of Bargen) isolated from the focal and local lesions of cases of uncomplicated chronic ulcerative colitis (*Collected Papers Mayo Clinic*, 1928, XX, 230); and *Puerperal Septicemia Antistreptococcic Serum* (refined and concentrated) (*Amer. J. Obst. and Gyn.*, 1929, p. 297) prepared from the blood of horses immunized against the specific toxins of virulent cultures of hemolytic streptococci isolated from cases of puerperal septicemia (*Brit. Med. J.*, 1931, *I*, 350). The 'S. C. A.' Serum is supplied not only as a concentrated equine serum, but also as an unconcentrated bovine serum, the latter to be used for patients who are sensitive to horse serum proteins.

USES

● As in the general rule of serum therapy, the good effects of these streptococcic antiserums are in proportion to the size of the dose and the early injection of the preparation. The polyvalent hemolytic streptococcus antiserums are administered intramuscularly or intravenously in doses of from 50 to 100 cc., this dose being repeated at 8 to 12 hour intervals or at more frequent intervals in severe cases, until improvement is noted. When using concentrated Polyvalent Antistreptococcic Serum, one-fifth of the dose as used for the unconcentrated product is advocated. The Antistreptococcic Serum Poliomyelitis (*J. A. M. A.*, 1921, *77*:588) (*Arch. Ped.*, 1934, *51*:11)

is administered intramuscularly or intravenously in doses of 5 cc. to 20 cc. and repeated at 12 to 24 hour intervals, until a total of 40 to 50 cc. of antiserum is used. S-C-A Serum Concentrated (Small, J. C.: *Med. Clin. of N. A.*, Sept., 1931) is administered intramuscularly in daily doses of 5 cc. or 20 cc. of the unconcentrated serum, all serum to be given within the first four days of treatment. The Ulcerative Colitis Antistreptococcic Serum is employed intramuscularly or intravenously in initial doses of 1 cc. giving daily injections, or administration is on alternate days in gradual increasing doses up to 5 cc.

Recent reports on the value of commercial streptococcus serums in hemolytic streptococcus infections reveal that their value is not significant (*New Engl. J. Med.*, 1934, 211, 99); (*J. Pediatrics*, 1934, IV, 746; *J. Immunol.* May, 1935); (*Lancet*, *2*, 1367 (1934); *1*, 1085, 1112 (1935)). In many instances, there appears no justification for the use of these preparations. The use of the newer chemotherapeutic agents, sulfanilamide, sulfapyridine and related compounds, is more valuable in the treatment of hemolytic streptococcus infections.

ANTIPLAGUE SERUM

Antiplague Serum (Serum Antipestis; Yersin's Serum)—This is the blood serum obtained from animals immunized against *Pasteurella (Bacillus) pestis*. Several techniques differing in the details of their mode of preparation are employed for preparing the several marketed antiplague serums. By injecting horses with filtrates of *P. pestis* cultures or even extracts of the organisms containing bacterial protein, the serum produced is antiendotoxic as well as antibacterial. Such a preparation has been used in the treatment of bubonic plague (*Indian J. M. Research*, 1930, 17). Prompt intravenous administration of very large doses (up to 100 cc.) of antiplague serum is claimed by some workers to be of great value in the treatment of bubonic and even the pneumonic type of plague. The dosage is repeated daily, until an improvement is noted. Intramuscular injections of 20 cc. are advocated as the prophylactic dose. The English Plague Commission has however reported that up to the present, serum therapy has not proven efficacious in the treatment of plague. Naidu and Mackie (*Lancet*, 1931, II, 893) have reported encouraging results with a serum prepared by immunizing cattle by a special technique.

ANTIGONOCOCCIC SERUM

Antigonococcic Serum is prepared from the blood of animals which have been immunized against many strains of representative types of gonococci. The potency of the finished preparation is gauged by determining the content of complement-fixing bodies. This preparation is recommended by some workers to be used early and to be administered intravenously or at least intramuscularly in large doses (30 cc. to 50 cc.) in gonococcus bacteremia or in certain of the complications following gonococcic infections (as apididymitis, orchitis, salpingitis and other local complications and in arthritis, endocarditis, etc.). Ferguson and Associates (Repr. No. 28, *Ven. Dis. Inform., U.S.P.H.S.*) report interesting findings on a study of the comparative value of stock vaccines, convalescent serum and commercial antigonococcal serum.

OTHER MARKETED IMMUNE SERUMS

ANTIBACTERIAL serums other than mentioned are being presented to be used for the prevention and treatment of disease. Anticholera Serum (Harvey, W. F., 1929, *A System of Bact.*, IV, 421, London), Antipertussis Serum, Antibrucella (Antimelitensis) Serum Polyvalent (Melitensis and Abortus) (*Deut. med. Wochschr.*, 1934, v. 60, 788), Antistaphylococcus Serum (*J. Path. and Bact.*, 1933, XXXVI, 87), Tularemia Antiserum (*J.A.M.A.*, 1933, *101*:1433) and others are in use. The results of many of these products have been unsatisfactory, discouraging or indeterminate. In other instances, sufficient evidence has not been made available to warrant their wider use. Many workers and especially Rodet (*Lyon Med.*, 1931, 201, 205) have prepared and used antityphoid serum. Recent clinical trials with a new antityphoid serum have proven of value in the treatment of typhoid fever. (*Lancet*, 1935, *228*:799, 1095). (*Brit. Med. J.*, II, 238, 1118, (1937)). Antityphoid serum has also been advocated as an efficient prophylactic agent (*Brit. Med. J., I,* 1090, 1091 (1938)). Serum from immunized animals have been tried in measles therapy, (*Lancet*, 1927, I, 93). Wolff and Associates (*Proc. Staff. Meetings Mayo Clinic.*, 1933, VIII, 634) review recent attempts to develop serum effective against cancer. Favorable reports were presented on the use of cowpox serum in the treatment of whooping cough in children (*Rev. med.latino-am.*, 22, 521, (1937)). There is of course the constant experimentation in the attempt to develop efficient serum therapy for all diseases affecting mankind and even lower animals. The next decade will undoubtedly bring forth many advances in serum therapy.

XII

IMMUNE HUMAN SERUM

WHILE one of the lower animals (horses, mules, cattle, goats, rabbits, etc.) may be utilized to obtain a serum containing antitoxic, agglutinating, bacteriotropic (immune opsonins) or bacteriolytic antibodies for the specific microorganism, immune human sera are sometimes employed as prophylactic agents or in the treatment of specific diseases. Such serums are obtained from convalescent cases of those recovered from the specific diseases. The etiological agents of the latter are usually not known or not available, and it is, therefore, impossible to prepare artificially immune serum by using the lower animals. Complete protection by the use of convalescent sera is of short duration, lasting only a few weeks.

IN MEASLES

● Successful results in preventing measles (Meader, F. M.; 1935, *Am. J. Nursing, 35,* 199), and mumps (*"A System of Bact.,"* 1930, H. M. Stat. office, VII, 273, London) by convalescent human serum, employing 5 cc. doses (intramuscularly) for children up to 4 years and in doses up to 10 cc. for older children have been obtained. Due to the difficulty in obtaining convalescent serum for immunizing purposes, Degkwitz (*Z. Kinderheilk.* 1920, XXV, 134), suggested the use of normal adult blood serum on the assumption that almost 90% of all adults had had measles. Later there was suggested the use of placental blood obtained a few minutes after the delivery. Dulitskiy (*Jr. Po Rannemu Detskomu Vozrastu:* 1932, XII, 322) reported on the extensive use of this type of serum treatment in measles. Karelitz (*Proc. Soc. Exp. Biol. Med.*, 1934, XXXI, 793) used the globulin extract of immune adult serum in the prophylaxsis of measles. Researches by McKhann and Associates (*Am. J. Dis. Child.*, 1933, XLV, 475) (*J.A.M.A., 109,* 2034, (1937)) of the pediatric dept. of Harvard Medical School have resulted in the production of a marketable preparation ***Immune Globulin (human) (Placimmunin).*** It is a refined concentrated globulin extract prepared from the human placenta, containing measles-immune bodies or factors of the type transmitted from the mother to the fetus. It is administered intramuscularly in doses of from 2 to 10 cc. as a prophylactic agent. Levitas (*J.A.M.A.*, Aug. 1935, *105:*493) concludes that "Immune Globulin (Human) is of definite value in treatment, modification and protection against measles, and of especial value in young children", though an editorial in the same issue of the journal concerning this product states:

"its present status does not seem to warrant general use." For further information concerning the use of human serum in measles, see *Brit. Med. J.*, 1931, I, 12; II, 977; 1932, 183; II, 260; 1937, II, 612; 1938, I, 1003. Karelitz (*Jr. Pediat.*, *13*, 195 (1938)) presents an evaluation of measles prophylactic agents.

Placimmunin or Immune Globulin (Human) is apparently equal to convalescent serum in its usefulness. It possesses the advantage of easy and universal availability. The method of standardizing this product gives an added advantage of presenting a preparation of known potency. In one of the techniques, this biologic product is standardized for immunizing potency by measuring the diphtheria antitoxin titre of the placental blood from which it is prepared. Such procedure appears satisfactory as an adequate basis for determining the measles-immunizing potency of Human Immune Globulin.

In another technique, Immune Globulin (Human) is standardized by determining the total nitrogen (by the Kjeldahl Method), the final product containing at least 10 mg. of nitrogen per cc. Severe or general reactions are infrequent but local reactions may prevail. Briefly presented, the methods for preparing placental extract (*J. Inf. Dis.*, *52*, 268 (1933)) is to extract with saline solution the finely ground organs. The centrifuged extracts containing fetal and maternal blood serum and placental tissue protein are treated with ammonium sulfate to one-half saturation. The precipitated globulins are dialyzed to remove the ammonium sulfate. After filtration, through paper pulp, a super-centrifuge and then through Berkefeld filters, a preservative is added, sterility tests are performed, the preparation is standardized and tests are made in animals and humans.

In a review of the literature, one must conclude that convalescent serum, adult serum and especially Immune Globulin are of value in the prevention and modification of measles. As prophylactic agents, large doses should be given early in the period of exposure, preferably before the fifth day of contact. The Council on Pharmacy and Chemistry of the A.M.A. discusses the present status of Immune Globulin (Human) (*J. A. M. A.*, *111*, 1764 (1938)) and Holroyd (*West Va. M. J.*, *35*, 121 (1939)) presents a review with an extensive bibliography on the prevention and modification of measles with various types of blood and immune globulin.

IN STREPTOCOCCIC INFECTIONS

● Convalescent serum, plasma or whole convalescent blood (preferably pooled), obtained from patients who are two or three weeks convalescent from scarlet fever, have been injected in doses of from 40 to 200 cc. in the treatment of early toxic cases of scarlet fever with encouraging results, (*Arch. f. Kinderheilk*, May 6, 1932, p. 129) (*Military Surgeon*, *81*, 355 (1937); *Bull. N. Y. Acad. Med.*, *14*, 361 (1938). Blood serums from convalescent patients of various streptococcic infections have been advocated in the treatment of similar or closely related infections caused by streptococci (Baum; *J.A.M.A.*, 1934, vol. 103, 2062). Human convalescent scarlet fever serum is advocated as a prophylactic for individuals exposed to scarlet fever (*Wisconsin M. J.*, *37*, 821, (1938)). Adequate amounts of scarlet fever convalescent serum injected intravenously appear to give rapid and satisfactory results (*J. Pediat.*, *14*, 256 (1939)). The use of sulfanilamide in addition to seroprophylaxis resulted in no reactions whereas convalescent serum alone produced reactions (*J. Pediat.*, *14*, 270 (1939)). Toomey (*Ohio State M. J.*, *34*, 1213 (1938)) states that "there is no good reason passively to immunize individuals exposed to varicella, erysipelas,

whooping cough, mumps, encephalitis, and scarlet fever with human convalescent serums." Even though below are listed references which favor this therapy, the evidence as a whole is inconclusive. Toomey's advice appears valid as to the use of convalescent serum or adult whole blood as either prophylactic or therapeutic agents in the diseased conditions mentioned.

IN OTHER DISEASES

● The use of convalescent serum from one recovering from typhus fever has been employed with but little success in the prophylaxsis and treatment of typhus fever (*"A System of Bact.,"* H. M. Stat. Off., 1930, VII, 393, London). Nicolle (*Rev. d'Immunol.*, Jan. 9, 1935) reports the successful use of murine virus, the activity of which has been reduced by the addition of bile, in immunization against typhus fever in Morocco though he further advises that the best treatment is the employment of convalescent serum. Epidemic or Infectious Jaundice (Weil's disease) has been treated successfully with the blood of convalescent cases (*J. Exp. Med.*, 1918, XXVII, 283). The use of convalescent serum (*"Poliomyelitis"* (Internat. Comm.), 1932, Baltimore) or whole blood (for prophylactic purposes) has been employed for protection against and treatment of poliomyelitis (*Brit. Med. Jour.*, 1933, I, 132), (*J.A.M.A.*, *109*, 402 (1937)) though experimental evidence recently introduced points to its ineffectiveness (*J. Pediatrics*, 1935, *6*, 615), (*J. Immunol.*, 1935, *28*, 345).

Intramuscular and intravenous doses of from 40 to 80 cc. and intraspinal doses of from 10 to 20 cc. of immune human serum (obtained from cases which have passed through an attack of poliomyelitis) have been employed in the treatment of this disease (*J. Pediat.*, 1934, V, 9). Girdlestone (*Lancet*, *235*, 642 (1938)) advocates the use of convalescent serum without delay and in massive doses.

Gunn (*Brit. Med. Jour.*, 1932, I, 183) advises the use of convalescent serum as a prophylactic against chicken pox. Post-vaccinal encephalitis has been treated with convalescent and normal human serum (*Report Comm. on Vaccination*, London, 1930, Ministry of Health, H. M. Stationery Office) and Ledinghand and Associates (*Brit. J. Exp. Path.*, 1931, XII, 357) report on the preparation and content of antival body of a specific serum prepared from the horse. Convalescent serum was successful in checking an outbreak of mumps (*Am. J. Dis. Child.*, 1931, XLII, 1109). Hyland (*Nebraska M. J.*, *22*, 342 (1937), *Rocky Mountain M. J.*, *35*, 132 (1938)) advocates the use of convalescent serum in many acute contagious diseases.

Szep (*Dermatol. Zeit.*, *76*, (1937)) reports favorably on the use of human antigonococcal serum in the treatment of complicated gonorrhea cases. Convalescent sera have also been used in yellow fever, psittacosis, and in the treatment of staphylococcic and streptococcic septicemias.

Convalescent human serum has been used in the treatment of cases of plague (*Indian Med. Gaz.*, *72*, 469 (1937), and actinomycosis responded to therapy by specific convalescent serum (*Wien. klin. Wochschr.*, *50*, 1176 1937)).

Due to the beneficial results with some convalescent serums, many investigators have recommended the establishment of agencies for collecting and distributing these convalescent serums, as the latter are not obtainable from commercial manufacturers. At present the bacteriological laboratory in the hospital is usually called upon to collect the blood and prepare the immune serum for administration.

REQUIREMENTS FOR SHIPPING BIOLOGICAL PRODUCTS FROM HUMAN SOURCES

● Convalescent serum or whole blood to be shipped in interstate commerce throughout the United States must meet certain requirements, designated as the "Minimum Requirements for Biological Products from Human Sources", promulgated by the National Institute of Health as of November 23, 1934. Among these regulations are the following:

"Only those persons may serve as a source of biologic products who are certified by a licensed doctor of medicine as being free of disease transmissible by blood transfusion as far as can be determined clinically, including personal history, physical examination, and the blood Wassermann reaction, upon the date upon which the material is obtained from the individual."

"An individual serving as a source of a biologic product shall be known to be free of transmissible disease for a period of 8 days following the date upon which such material was obtained. Only those individuals may serve as donors of blood for the manufacture of convalescent serum who are certified by a licensed doctor of medicine as having had an attack of the disease against which such immune serum is desired."

"A donor of blood for convalescent serum shall have had no other transmissible disease from the onset of the disease against which immune serum is desired to the date of bleeding."

"The date of bleeding of a donor of convalescent serum shall not be more than 90 days after the date of onset of the specific illness. The individual shall not serve as a donor until after an afebrile period of 7 days, during which there shall be no active clinical manifestations of disease, and in the case of scarlet fever not sooner than the 20th day after onset."

"All requirements for methods of manufacture and distribution, including sterility tests, labeling, dating, and records of distribution shall be those for other biologic products as required by the Treasury Department under the Biologics Act, dated July 1, 1920, except that all biologic products from human sources should be passed through a filter efficient against known pathogenic bacteria, and except that the finished product should contain an acceptable preservative, unless a sufficient number of the final containers are tested to insure sterility of the product."

IMMUNOTRANSFUSION

THE use of compatible human blood (blood transfusion) in severe prolonged infections with anemia has often been found to turn the tide in favor of recovery, especially in the treatment of streptococcus septicemia. Wright (*Lancet*, March 29, 1919) advocated the addition of vaccine to the blood and after a suitable interval, the whole mixture is injected into the patient. He called this method "immunotransfusion." Other methods have been introduced in which streptococcal infections were treated, these procedures designated also by the same term (*J. A. M. A.*, *98*, 2191 (1932); *100*, 100 (1933)); (*Am. J. Dis. Child.* *33*, 116 (1927)); (*Lancet*, *1*, 391 (1934)). In these methods, the suitable donor is given a single injection of a vaccine a few hours before the withdrawal of blood; or the suitable donor may have received a series of injections of a vaccine prepared from the organism

obtained from the patient; or the donor receives a series of injections of vaccine and a few hours before the withdrawal of blood an injection of some non-specific substance capable of producing a marked leucocytosis is given. These methods when used are generally employed as a measure of last resort. They can not be carried out with ease and though favorable results in isolated cases have been reported, they are not widely used nor recommended generally. A technique for immunotransfusion which appears to be based on sound immunologic principles has been suggested recently by Lyons (*J. A. M. A., 105,* 1972 (1935)). He recommends the selection of donors whose serum contains opsonins for the organism infecting the patient. Such donors of a compatible blood group with the patient are tested and only those whose serum promotes active phagocytosis for the organism causing the infection are used. This or any of the other methods are advocated only in cases of severe septic infection where other procedures are inadequate.

PLAIN, NON-IMMUNE SERUM

N*ormal serum* from humans, but usually from different animals, especially the horse, though not a specific immune product, for it does not necessarily contain immune antibodies, has been employed in "non-specific therapy" and in the treatment and as a preventive of hemorrhage to increase the coagulability of the blood. The serum for marketable purposes is usually obtained from a normal animal under sterile precautions. The product is passed through a Berkefeld filter; sterility and toxicity tests are performed; and it is then marketed with or without a preservative in sterile containers.

Wherever possible, human serum or defibrinated human blood should be used for humans. Normal sera are occasionally employed in the treatment of some diseases. In some cases it may be that the normal serum contains natural antibodies specific for the disease for which it is used, as in the use of sera of cattle in the treatment of human anthrax (advocated in Argentine). In most instances, where it is used in the treatment of certain skin and other diseases, normal serum probably owes its efficacy largely to non-specific factors (see non-specific protein therapy). There is also the possibility that normal human adult serum may contain antibodies specific for the etiological factors of diseases commonly found among children, so that this may account for its value as a prophylactic agent against some diseases.

Normal Horse Serum (Serum Equinum) has been applied locally to bleeding surfaces or to accessible mucous membranes. Sterile cotton saturated with the sterile serum is applied and repeated hourly until the hemorrhage is controlled. In the bleeding from gastric or duodenal ulcers, 30 to 100 cc. doses are given by mouth and repeated if necessary at intervals of from 12 to 24 hours. In certain cases, hemorrhages of the lower bowel may be arrested by giving normal serum as an enema. In bleeding from the uterus, the normal serum is applied as a uterine pack. Hamon and Pineau (*Bull. Acad. med.,* 641, Nov., 1934) advocate the employment of very small intramuscular injections of horse serum into the gums and local application as a hemostatic in hemorrhages following dental extractions. They believe this method is of value for all hemorrhages of a sufficiently accessible

site such as those following excision of tonsils, nasal growths and even in uterine hemorrhages.

Normal horse serum has been and is employed to aid the healing process in the treatment of burns caused by fire, hot water, or acids. The serum is applied by spraying it on the burned area, employing a warm saline bath before each application. This treatment is repeated at least twice daily. After applying the serum, rubber tissue is used to protect the wounded area and to prevent evaporation, thus insuring prolonged action of the serum. Subcutaneous or intramuscular injections (preferably the latter) in doses of from 20 to 30 cc. or intravenous injections of from 10 to 20 cc. in cases of extreme urgency are given in the treatment of hemophilia and other forms of hemorrhage from wounds and fractures, or during or following surgical operations, in persistent epistaxis appearing as a general oozing, in melena neonatorum and in purpura hemorrhagica. The dose may be repeated daily until a total of 100 cc. of serum has been administered. Preoperative injections with three or more doses of Normal Horse Serum are practiced by some before performing surgical operations upon hemophilics. The usual precautions to prevent allergic reactions or anaphylactic shock must be taken not only during parenteral administration but also before topical applications are given as in open wounds or on burns.

This product diluted 1:10 or the marketed diluted preparation is employed to determine hypersenitiveness to the proteins of horse serum (so-called serum sickness (which see)).

XIII

ANTIGENS USED AS THERAPEUTIC AND IMMUNIZING AGENTS

ANTIGENS (meaning antibody-producers) are substances possessing the power or capable of inducing the formation of specific antibodies. Antigens whatever their composition may be, are used for the production of active immunity, which gives a protection for a considerable length of time (in some cases for years) as compared with the action of therapeutic sera which are of avail only for a few weeks. The antibodies produced though not immediately available last however for longer periods of time. The most important antigens used as medicinal agents are modified smallpox virus and antirabic virus, the bacterial vaccines, immunizing toxins, toxoids, and tuberculins.

You will note that the antigens are of various types. Smallpox and rabies antigens (or viruses) are attenuated by passage through some animal (bovine species). Bacterial antigens (or vaccines) are generally attenuated or killed by heat or chemicals. Occasionally, attenuation of bacterial vaccines may be effected by growing the organism under unfavorable conditions (as B. C. G. used in immunization against tuberculosis), or by autolysis (autolyzed pneumococcus antigen and bacteriophage filtrates), or by admixture with an immune serum (activated vaccines). In other instances the metabolic products of bacterial growth or preparations of such products serve as the antigens, as in the case of immunizing toxins, toxoids, and some of the tuberculins.

VACCINES

THE terms vaccine and vaccination (from the Latin *vacca*, cow) had their origin back in 1796, when Dr. Jenner inoculated (vaccinated) individuals with vaccinia (material from cowpox, the cow disease) and successfully produced an immunity against smallpox. Since then many products used in the immunization of humans have been termed *vaccines*.

The term "vaccine" employed in a broad or general sense, as it is used most frequently today, may be defined as an infective agent so treated or modified as to prevent it from growing and causing disease in the body.

It is not, however, changed so as to affect or prevent the production of specific antibodies or protective substances when injected into the body. The term is most frequently used in a specific sense when referring to bacterial vaccines.

The term *"bacterial vaccines," "bacterial antigens,"* or *"bacterins"* is, however, to include only those products which are suspensions of attenuated or killed bacteria in physiological normal salt solution, usually preserved with 0.5 per cent phenol, 0.2% to 0.4% cresol, 50 per cent glycerin, organic mercury compounds, or a similar effective preservative.

Bacterial vaccines are divided into two main classes: (a) *stock* and (b) *autogenous bacterial vaccines.* Autogenous bacterial vaccines are those prepared from the patient's own infection. The offending organism or organisms obtained from the lesion or infected part of individuals are used for the preparation of the bacterial vaccine, which is to be later reinjected into them. Stock bacterial vaccines are those prepared from stock cultures kept in the laboratory and consisting of strains capable of stimulating the body to immunity production.

A *simple bacterial vaccine* is one made from only a single species of microorganism, as plague vaccine, made from *Pasteurella* (*Bacillus*) *pestis. Mixed bacterial vaccines* are those containing two or more different species of bacteria, as triple vaccine, made from typhoid bacillus, paratyphoid A bacillus and paratyphoid B bacillus. A *monovalent bacterial vaccine* is one containing but a single strain of the particular organism or organisms. *Polyvalent bacterial vaccines* are those prepared from numerous strains of the particular organism or organisms. These are obtained from many different sources.

WHEN USED

● Bacterial vaccines are used for the production of active immunity (*Brit. Med. J.*, 1926, *I*, 815) and are employed mainly:

1. For prophylactic purposes, (resulting in specific antibody production). Prophylactic or preventive immunization by using bacterial vaccines may be:

(a) To create an immunity against a primary infection, especially against diseases which are endemic in particular areas or to which an individual may be susceptible, such as typhoid fever, cholera, plague, etc. This is the greatest use to which bacterial vaccines are applied. A report of the Committee on Immunization including vaccination of the British Medical Assoc. (supplement to the *Brit. Med. J.*, June 22, 1935, p. 282) states that "Disease can be eradicated by mass immunization."

(b) To immunize against possible secondary infections, especially against those diseases which most frequently complicate primary infections, as pneumonia following grippe or influenza or streptococcic infections following severe cases of tonsillitis, scarlet fever, etc.

(c) To immunize against infections that usually set in following wounds, surgical operations, etc.

2. They are also used in the treatment of focal, chronic, and in acute localized infections.

3. In the treatment of so-called "human carriers."

4. Attempts are being made by some to use bacterial vaccines in the early stages of acute infections. In many cases good results have been reported. It may, however, be advisable to await further clinical evidence of the value of such treatment, before its general use for this purpose is to be advocated.

The therapeutic effects of bacterial vaccines (Dudgeon, "*Bacterial Vaccines and Their Position in Therapeutics,*" 1927, London) are probably due to both specific and non-specific agencies (Wright, *Lancet,* March 29, 1919 and 1931, *II,* 225, 227, 233). Even in acute infections or in generalized infections, their administration may stimulate the immunizing capacities of the skin or other tissues capable of greater immunologic response. (For a full discussion and bibliography, see W. G. Liston, et al., *Tr. Med. Chir. Soc. (Edinburgh),* 1926-27, *XL,* 101 and W. F. Peterson "*Protein Therapy and Non-Specific Resistance,*" 1922). Armstrong and Harrison (Repr. No. 1576, *U. S. P. H. Reports*) recently presented interesting experimental evidence in the heterologous experience in immunization as a factor in the resistance to disease.

Stock bacterial vaccines, which are always polyvalent, are generally employed for prophylactic purposes, inasmuch as autogenous bacterial vaccines are not available for immunizing individuals who have no lesion or infection, from which cultures can be made for the preparation of a bacterial vaccine.

INOCULATING LIVING MICROORGANISMS

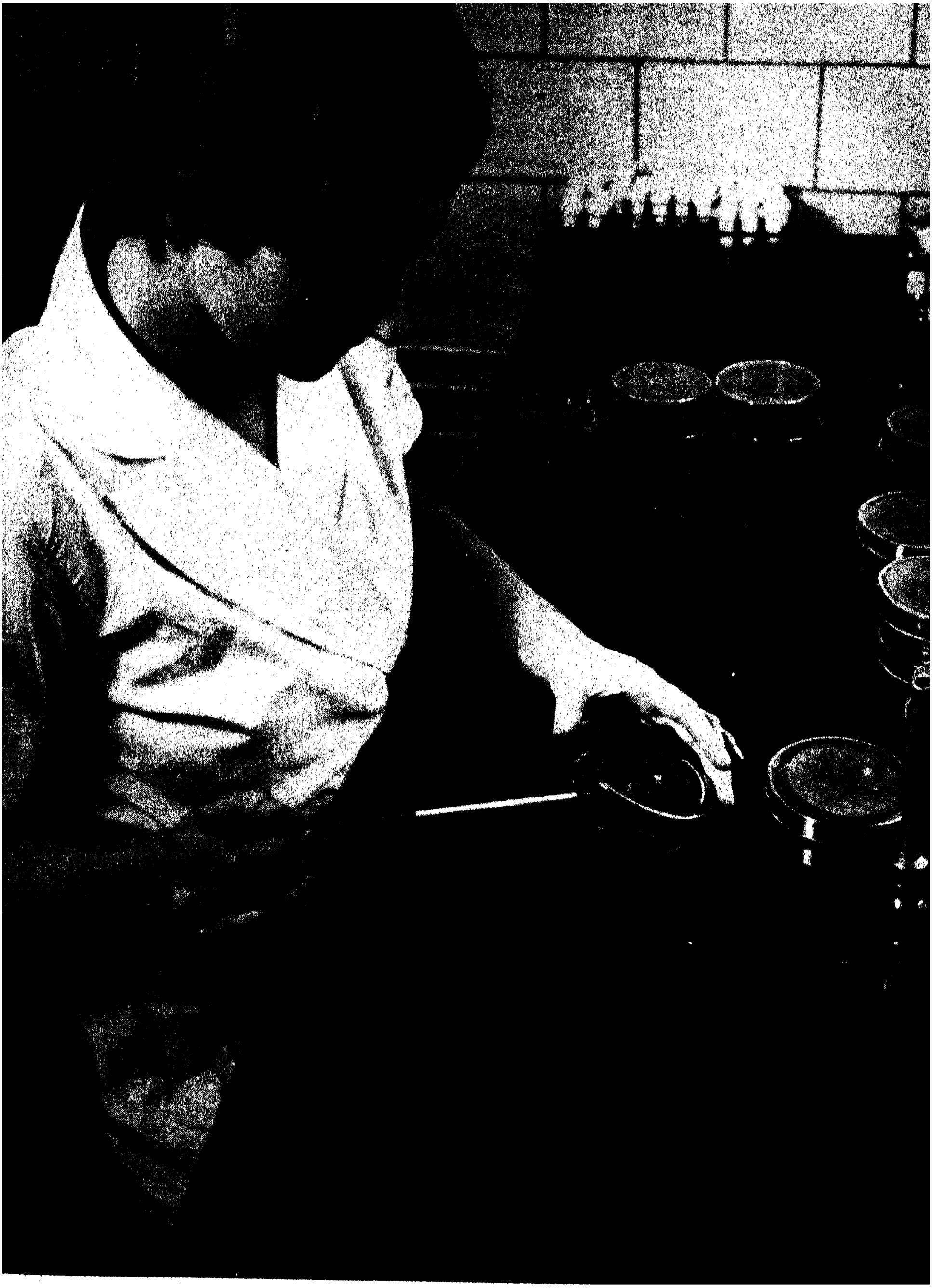
● In some foreign countries prophylactic immunization has been attempted by employing living instead of attenuated or killed bacteria. The use of the latter are as yet preferred, as they are safer and more dependable, and the tissue cells are stimulated to produce antibodies just as if the organisms were living (*Brit. Med. J.,* 1915, *II,* 629). Smirnow and Associates (*Sovet. vestnik Venerol. Dermatol.,* 1935) report that of all the existing methods for vaccine therapy in chronic gonorrhea, the injection of living cultures of gonococci gave the best results.

The element of safety in the use of killed bacterial vaccines has resulted in the extensive use of this type of vaccine in preference to other types.

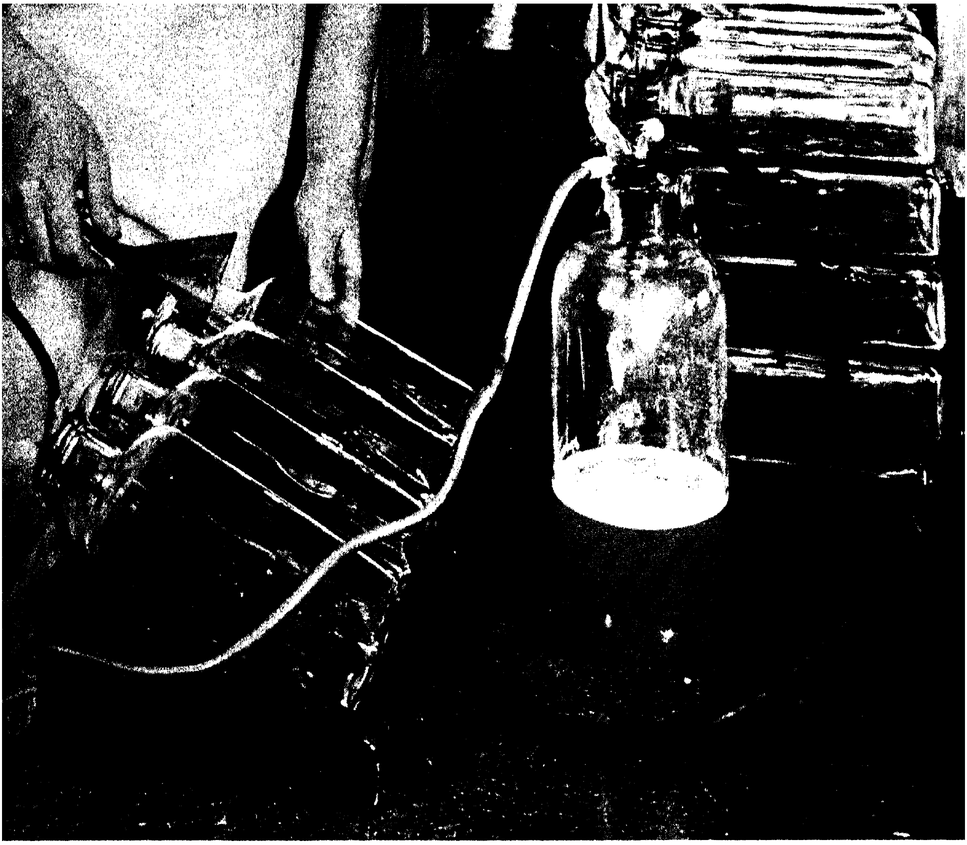
PREPARATION OF BACTERIAL VACCINES

● The first step in preparing bacterial vaccines is to obtain a culture medium most suitable for the growth of the organism or organisms from which the vaccine is to be prepared. A solid medium is generally used, plain agar being satisfactory in many cases, while in other instances a solid culture medium containing natural fluid as blood agar may be necessary. In preparing autogenous vaccines the preliminary examination of a stained smear of the material will aid in choosing the proper medium.

IN preparing autogenous bacterial vaccines the material from the lesion or infection is collected under sterile conditions. The pyo-sero-culture of Wright (*Brit. Med. J., II,* 629 (1915)), extended by Solis-Cohen (*Brit. Jour. Exp. Pathol., VIII,* 149 (1927)), in which the patient's blood is used as the culture medium has been employed by some as the culture method of choice for obtaining the infecting organism for autogenous preparations. In the pathogen-selective method of Solis-Cohen, the material from the suspected focus of infection is spread with sterile swabs on the bottom of a sterile test tube and therein is placed 3-5 cc. of the patient's blood collected under aseptic conditions. Allow to clot and incubate for 24 hours. Plate on blood agar. Identify pure colonies and proceed to prepare the autogenous vaccine with the organisms isolated. In preparing an autogenous vaccine, the technique and choice of culture media used are to be so arranged as to secure growth of the bacteria of primary importance rather than the bacteria of



Selecting a colony of bacteria of known origin preparatory to bacterial vaccine manufacture.



Harvesting living organisms preparatory to bacterial vaccine manufacture.



Purity tests for bacterial cultures.

only secondary infection. If the material reveals a mixed infection, judgment must be employed in selecting the bacteria to be used in preparing the vaccine. Contaminants as *B. prodigiosus* and spore-bearing bacilli as *B. subtilis* are not to be included. Cohen's pathogen selective method is regarded by some workers as aiding in the selection of bacteria for the preparation of vaccine in mixed infections on the assumption that only those bacteria which develop and grow in the whole coagulated blood of the patient are apt to be virulent. This thought however does not take into consideration the fact that an organism unable to grow in the blood or even produce a septicemia may still be able to produce local infection. It is on this account that this method is not widely practiced.

Intracutaneous skin tests have also been advocated for the selection of those bacteria in a mixed infection which are to be used in the preparation of the autogenous vaccine. The thought is that only those bacteria giving positive skin reactions should be used. The status of such procedures and the exact value of the method are as yet unknown and difficult to assess.

For stock bacterial vaccines in the main polyvalent strains of the organism or organisms are isolated in pure culture and used. In mixed bacterial vaccines the individual organisms are obtained or isolated and secured in pure culture and then cultured separately to obtain sufficient organisms for the preparation of the vaccine. The suspensions of the different species are later mixed together. Freshly isolated organisms are almost invariably of the "S" type (smooth colony variation when cultured) but in continued subculture tend to degenerate into the "R" type (rough variation). "R" variants have been found to be valueless as protective antigens and being valueless as immunizing agent they must therefore be excluded in the preparation of vaccines (*Jour. Path. and Bact., XXIV*, 36; *XXIX*, 318; *XXX*, 345, 566) (*Lancet, I*, 1337; *II*, 963 (1929)). Stock vaccines should therefore be made of recently isolated cultures (see below) and where this is impracticable, the cultures should be kept so that they retain their full antigenic value. Wide mouth rectangular bottles, so-called Blake Bottles, and Kolle flasks are employed most frequently for mass cultures in the preparation of stock vaccines, bacterial antigens, etc. On a small scale, slants are made in tubes or flasks or small rectangular bottles may be used.

Generally, the cultures are allowed to incubate for twenty-four hours at body temperature (37°C.). Young cultures are most desirable for preparing bacterial vaccines, but occasionally it may be necessary to extend the incubation period for forty-eight to seventy-two hours or until growth becomes apparent. This is especially true in preparing autogenous and stock bacterial vaccines from some of the higher forms of bacteria. At the end of the particular period of incubation the bacteria are procured under aseptic conditions by pouring sterile physiological normal salt solution on the solid media, gently shaking or scraping (with a sterile platinum needle) until all the microorganisms have been suspended. If scraping is necessary, avoid removal of any of the culture medium. If bouillon or other liquid medium was used, the culture is centrifuged at high speed, the supernatant liquid discarded and the sediment containing the bacteria is suspended in sterile saline solution, shaken well, again centrifuged, the supernatant washings discarded and the sediment again resuspended in sterile saline solution. The salt solution suspension of bacteria in either case is examined by stained smear for purity and is then poured into a sterile flask, bottle or tube with or without sterile beads, shaken by some

mechanical device or by hand (on a small scale) so as to break up the clumps, and a uniform emulsion is made. Rubbing in a sterile mortar or grinding may occasionally be necessary when making vaccines of fungi. Bacterial vaccines are standardized by estimating the number of bacteria in each cubic centimeter of suspension (see below).

After the number of bacteria per cubic centimeter has been determined the suspension is diluted by adding of sterile physiological salt solution, so as to bring the content to 1,000,000,000 per cubic centimeter or to any other content desired. The suspensions are now sterilized by treatment with a bactericide or by heat in a water-bath at the minimum temperature and time of exposure that will kill the bacteria, which in most instances is 60°C. for one-half hour to one hour, and then cresol (0.2 to 0.4 per cent) or other suitable preservative is added. Some workers dilute their suspensions after sterilization with normal salt solution containing cresol, so called cresolated normal salt solution. The bacterial vaccines are now filtered through sterile filtering paper, the filtrates subjected to the purity and safety tests (see *General Considerations of Biological Products*) and the finished preparation is placed in sterile containers (either ampuls, vials or small bottles stoppered with rubber caps, or syringes).

Chemically-killed bacterial vaccines are advocated by some workers as being more antigenic than heat-killed preparations. They claim that the heat (56°C. to 60°C.) used in sterilizing bacterial vaccines destroys part of the antigenic value of the latter and that this is especially applicable to those organisms producing toxins, as the latter are readily inactivated by heat and not by chemicals. Phenol, cresol, and in France, ether, iodine and sodium fluoride, have been used. Newer disinfectants have been employed successfully for sterilizing bacterial vaccines, the bacteria possessing a high antigenic effect (Beck and Wolff: 1933, *Zentr. Bakt. Parasitenk.*).

Tricresol, most frequently recommended, is added so as to yield a final concentration of 0.4% for sterilization and preservation. In the case of mixed vaccines, separate suspensions of each organism are treated in this manner and then mixed in desired proportions. The mixtures are placed in an incubator at 37°C. for 24 hours. Test for sterility. If not sterile, reculture. Usually, 24 hours at 37°C. are sufficient.

For more complete details for preparing bacterial vaccines, especially on a large scale, see under Typhoid Vaccine.

The treatment of bacterial suspensions with formalin (comparable to the preparation of toxoids), producing the so-called "*anacultures*", and their successful use as immunizing agents has been reported (*J. Infect. Dis.*, *37:* 125; *43:* 189).

STANDARDIZATION OF BACTERIAL VACCINES (Determining Bacterial Content)

THE enumeration of bacteria in this or any other bacterial vaccine may be made by: (a) Direct count after dilution in a counting chamber (or still better in a specially constructed chamber (Helber chamber) for counting bacteria); (b) Indirect counting method of Wright (estimating number of bacteria in comparison with red cells in a fresh drop of blood, after a film of the mixture of both is stained); (c) Estimation of density or turbidity of the suspension by using nephelometers and by comparison with various standards (as McFarland's Barium Sulfate Standards); (d) Measuring the volume in the special centrifuge tube of Hopkins.

Counting Chamber Method

● In a small sterile test tube, place 0.1 cc. of the well agitated suspension and 4.9 cc. of sterile physiological saline solution. Use sterile pipettes and aseptic manipulation. Mix well; draw the suspension (diluted 1:50) up to the 0.5 mark in a leucocyte-counting pipette, and dilute up to the 11 mark with the following filtered freshly-prepared staining solution:

Saturated alcoholic solution of crystal violet	1.0 cc.
Freshly distilled water	10.0 cc.

Mix well by shaking for at least 2 to 3 minutes. Discard the first drop or two into a disinfecting solution and by capillary attraction fill a Helber counting chamber which had been accurately adjusted with a reinforced precision cover glass. The chamber and cover slip is first cleaned well with alcohol to remove adhering fat and dried to remove all particles. Allow the preparation to stand for 10 to 15 minutes. With a No. 6 objective and a No. 4 eyepiece, locate the center of the ruled field. If it is not possible to count with the 1/6 power objective, place a drop of thin cedar oil on the coverslip and use the oil-immersion lens. Count the bacteria in at least 20 squares, observing care in focusing on different levels for bacteria which may not have settled. All bacteria in clumps must be counted or accurately estimated. If the number of bacteria is too large, making it difficult to obtain a fairly accurate estimate or count, repeat the technique using a 1:100, 1:200 or 1:500 dilution of the vaccine instead of a 1:50. To obtain an approximate estimate of the bacterial count:

Divide the total number of bacteria in 20 squares, by 20, to obtain the average per square. The squares are 1/20 mm. by 1/20 mm. and the depth of the chamber is 1/50 mm., making the cubic capacity of each square 1/20,000 cu. mm. Multiplying the average per square by 20,000 will give the number by cu. mm. of diluted suspension. This multiplied by 1,000 will give numbers per cc. of diluted suspension, which when multiplied by 20 (dilution in white cell pipette) and by 50 (if the dilution of the original suspension was 1:50) will give the number per cc. of undiluted suspension.

$$\frac{\text{No. bacteria counted in 20 squares} \times 20{,}000 \times 20 \times 1{,}000 \times \text{dilution of suspension (50 if 1:50)}}{20} = \text{bacteria per cc. of suspension}$$

or

Average in each square multiplied by 400,000,000 will give numbers per cc. of diluted suspension.

A Petroff-Hauser bacteria counter may be employed instead of the Helber chamber. With a red cell pipette, the well agitated suspension is drawn up to mark 0.5 and diluted with the staining solution (given above) to 101, making a 1:200 dilution. Proceed from here on as given above after the mixture in the pipette is made. Either 10 or 20 squares are counted. The average per square multiplied by 200 and by 20,000,000 will give the bacteria per cc. of suspension used in the pipette.

Wright's Method

● In a hollow ground slide or watch glass crystal, mix thoroughly a drop of 1.5% sodium citrate in physiological saline solution, one drop of blood from a normal individual (or from one upon whom a red cell count is made) and one drop of the well-mixed bacterial suspension. Make several

thin films on clean dry slides. Dry in air and stain with Wright's stain. Examine with an oil-immersion lens. Note only those films which show bacteria and red cells properly stained and free from bacterial aggregates or clumps. Count the red cells and bacteria separately in a number of fields in different parts of the slide, until a thousand red cells have been counted. Note the number of bacteria counted at the same time and from the ratio of bacteria to red cells, the number of bacteria in one cc. of suspension is estimated. If the red cell count is performed the actual count is used, otherwise normal blood is estimated as having 5,000,000 cells per cu. mm., which means 5,000,000,000 per cc.

No. red cells counted : No. bacteria counted :: 5,000,000,000 : X = Bacteria per cc.

$$\frac{\text{No. bacteria counted} \times 5{,}000{,}000{,}000}{\text{No. red cells counted.}} = \text{Bacteria per cc. of suspension}$$

If the bacteria are too numerous in the various fields, the suspension is to be diluted with saline and the films are made with well mixed portions of the diluted suspension. In the calculation, the result obtained is multiplied by the dilution made to obtain findings in the original suspension.

Nephelometer Method of Measuring Suspensions

● This method is only to be used for suspensions in saline solution and which contain no coloring matter. For suspensions from broth cultures or other media apt to color such suspensions, nephelometric standards prepared with broth or coloring matter are used.

A measured quantity of the bacterial suspension usually 1 cc. is placed in a test tube of the same size and color as those used for the nephelometer standards. Dilute with a measured amount of saline to match the density in one of the standard tubes. Note the final dilution. To determine the approximate bacterial content, multiply the number of bacteria represented by the nephelometer standards which corresponds with the density of the bacterial suspension by the dilution of the bacterial suspension.

The nephelometric standards are hermetically sealed tubes of hard glass labelled serially from 1 to 10, each containing 1 per cent H_2SO_4 c. p., and 1 per cent $BaCl_2$, in the following amounts:

Tube	H_2SO_4 cc.	$BaCl_2$ cc.	Density corresponds with bacterial suspensions of about (millions per cc.)
1	9.9	0.1	300
2	9.8	0.2	600
3	9.7	0.3	900
4	9.6	0.4	1,200
5	9.5	0.5	1,500
6	9.4	0.6	1,800
7	9.3	0.7	2,100
8	9.2	0.8	2,400
9	9.1	0.9	2,700
10	9.0	1.0	3,000

Hopkin's Method

● **The number of bacteria is estimated from their bulk after centrifugalizing the bacterial suspension in a special tube for half an hour at approxi-**

mately 2,800 revolutions per minute. Before placing the suspension in the Hopkin's centrifuge tube, it is filtered to remove large clumps and bits of agar or other culture media. During centrifugation, the tube is covered with a sterile rubber cap or plugged with sterile cotton which is held in place by adhesive tape. A powerful centrifuge is required for the high speed used.

The Hopkin's tube is graduated to 1, 5, and 10 cc. and the narrow portion at the bottom is graduated in hundredths of a cc. During centrifugalization, the bacteria collect in the narrow portion. The supernatant fluid and all of the bacterial sediment above the 0.05 mark are removed with a sterile capillary pipette and 0.5% phenolated saline solution is added to the 5 cc. mark. This 1% bacterial suspension is well-mixed with a sterile capillary pipette. If the bacterial sediment does not reach the 0.05 mark, a correspondingly small amount of phenolated saline is added.

One per cent suspensions of the following species of bacteria yield the following approximate numbers:

	Millions per cc.
Diplococcus pneumoniae	2,500
Escherichia coli	4,000
Eberthella typhi; streptococcus; and gonococcus	8,000
Staphylococcus albus; and S. aureus	10,000

SELECTION OF CULTURES

● Mention was made previously that in the preparation of bacterial vaccines, typical smooth colonies should be selected, as these have been found to possess antigenic properties superior to the rough variants. In making stock bacterial vaccines, if smooth colonies are not found, it is possible to rejuvenate such bacterial strains by subculturing the organism several times in succession rapidly, alternately in broth (or suitable liquid medium) and in agar (or suitable solid medium) and then replating. Animal inoculation and recovery of the organism may be necessary at times.

IMMUNIZATION WITH BACTERIAL VACCINES BY MOUTH

● Various workers have prepared bacterial vaccines and have used them in specific cases by oral administration. Autogenous bacterial vaccines have been prepared and were used in respiratory infections (other than tuberculosis) and administered by mouth. The clinical results in these cases have not warranted advocating administration of bacterial vaccines by this method.

In a report (*Science, Aug.* 23, 1935) it appears that oral administration of bacterial vaccines may be of value as a prophylactic against colds and upper respiratory infections if large doses, administered frequently, on an empty stomach, are given. Dr. Besredka (*Bull. Inst. Past.*, 1930 *xxviii,* 49) advocated and used (and he still uses) immunization against the gastro-intestinal infections, especially typhoid fever (*Lancet* 1929, I, 1302), bacillary dysentery (*Ann. del. Inst. Past., 36:* 579) and cholera (*Leag. of Nat. Health Org.*, 1927, No. *C. M.*, 662 Geneva) by the oral administration of pills of the particular attenuated bacteria (see under *Vaccinum Typhosum*). A wider application of this method giving the so-called bili-vaccines, must be exercised before definite conclusions can be forthcoming as to the advisability of the universal advocacy of immunization per os in man against intestinal infections. See also under specific headings.

SENSITIZED BACTERIAL VACCINES OR SEROBACTERINS

(Antibody Antigens)

● To overcome some of the undesirable reactions the sensitized bacterial vaccines or serobacterins were introduced (*Ann. de l' Inst. Pasteur,* 1911, *XXV,* 193, 867; 1913, *XXVII,* 597, 607). These products are prepared in the same manner as the bacterial vaccines, but differ in the following additional steps:

After the bacterial suspension has been thoroughly emulsified, and the number of bacteria per cubic centimeter of suspension has been determined, a portion of the serum of an animal (which had been immunized against the same species of bacteria as in the suspension), is added. Though many employ suspensions of the living organism, others have advocated the use of attenuated organisms for sensitization by specific immune serum. The results are apparently just as satisfactory in most cases, and the danger of infection with living organisms is thus avoided. This mixture is allowed to stand for varying periods of time, usually twenty-four hours, in an incubator at body temperature, with occasional agitation. At the end of this time the mixture is centrifugalized and the supernating liquid (consisting of saline solution with excess of immune serum) is discarded. Normal salt solution is added, the mixture is shaken; and after centrifugalization, the supernatant liquid is again discarded. This treatment, which is repeated a few times, is done so as to remove any adhering serum. Enough cresolated physiological normal salt solution is finally added to give a bacterial content as may be desired. This is then sterilized and marketed after purity and safety tests have been conducted.

The treatment with immune serum is said to sensitize the bacteria so that they are more easily attacked by the forces in the patient's system. A portion of the immunity reaction which ordinarily would take place in the body (the breaking down of the bacterial cells) is produced in the test-tube during the incubation of bacteria and immune serum. The excess of the latter must be discarded, as is always done, inasmuch as it has been found to interfere with active immunization. It is believed that this reaction prepares the bacteria just as well as if our own systems were stimulated to prepare the antibodies for the disintegration of the organisms. Sensitized bacterial vaccines are therefore said to be more rapid in their production of immunity, especially since they give a degree of immediate protection, for the antibodies attached to the antigen are in part set free upon injection. They usually minimize undesirable reactions (*Arch. Int. Med.*, Nov., 1914). Larger doses of sensitized bacteria at more frequent intervals can be given with the production of a higher degree of immunity than it is possible to secure with non-sensitized bacterial vaccines (*Proc. Roy. Soc.*, Series B., *98;* 553). Sensitized bacterial vaccines of practically most of the bacteria (simple and mixed, and from which plain bacterial vaccines are prepared) are marketed. They are used in place of the plain bacterial vaccines, yielding results which are convincing as to their value.

DURATION OF IMMUNITY WITH BACTERIAL VACCINES

● The duration of an active immunity produced by injections of bacterial vaccines varies with both the individual and the specific antigen (bacterial vaccine) used. It might be emphasized that immunity following bacterial

vaccine immunization is not absolute. A lowered state of general body health and contact with large numbers of the specific infective agent may result in an infection with the particular organism against which an immunity was produced. Prophylactic treatment is not successful if sanitary precautions are to be disregarded by immunized individuals. The use of bacterial vaccines in the treatment of disease does not warrant discontinuance of any of the other forms of treatment. The latter are to be practised.

The actual duration of immunity by bacterial vaccine immunization is not definitely known. In fact, it is frequently not practical or difficult to determine. It may be as low as one month with some bacterial vaccines to one and even two years with others, rarely more. A good plan to practise is for susceptible individuals or those who are constantly exposed to specific bacterial infections to immunize themselves annually against the specific infective agent (bacterium). In many cases it is practical to keep up the antibody content by giving the initial series of injections first, to be followed every three to four months by a single injection (usually a large or massive dose).

BACTERIAL VACCINES AS PROPHYLACTIC AGENTS

THE following stock bacterial vaccines are used as prophylactic agents:

Cholera vaccine prepared from killed Vibrio cholerae (*V. comma*) is used with favorable results as an efficient prophylactic against cholera offering an immunity for from 3 to 6 months (*League of Nat. Health Organiz.* 1927 No. *C. M.* 662, Geneva). Three injections, the first ½ billion and the other two one billion bacteria each are usually given.

Meningococcic (Epidemic Meningitis) vaccine or Meningo bacterin prepared from killed meningococci (*Neisseria intracellularis*) is being employed as a prophylactic against epidemic cerebrospinal meningitis.

Pertussis (Whooping Cough) vaccine prepared from killed *Bacillus* (*Hemophilus*) *pertussis* is used as a preventive conferring protection against whooping cough.

Plague (Haffkine's) vaccine (Topley and Wilson, *Principles of Bact. and Immun.*, 1929, *II*, 1075, London), prepared from killed *Bacillus* (*Pasteurella*) *pestis* is used successfully for the prevention of (bubonic variety) plague, though the immunity lasts for a relatively short period.

Pneumococcic vaccine or Pneumo bacterin prepared from killed pneumococcus (*Diplococcus pneumoniae*) is used with some degree of success as a prophylactic agent against pneumonia. Felton and Associates (*J. Infect. Dis.*, 1935, *56*:101) report on the duration of the immunity following the use of pneumococcus vaccine and of various fractions thereof.

Streptococcus vaccine (prepared from all strains of killed streptococci) has been suggested as a prophylactic agent for the prevention of secondary streptococcic infections in scarlet fever and in other subacute and chronic diseases caused by the streptococcus.

Typhoid vaccine prepared from *Eberthella typhi* (*B. typhosus*) is used in immunization against typhoid fever ("A System of Bacteriology", 1929, IV, 15, London).

Typhoid combined or typhoid mixed vaccines —known also as:

Triple vaccine consisting of the typhoid (*Eberthella typhi*), paratyphoid A (*Salmonella paratyphi*) and paratyphoid B (*Salmonella schottmulleri*) bacilli are of recognized value as prophylactic agents for the prevention of typhoid and paratyphoid fevers.

BACTERIAL VACCINES FOR CARRIERS AND IN LOCAL AND CHRONIC INFECTIONS

● In the treatment of carriers or of local and chronic infections, autogenous bacterial vaccines are used wherever possible, stock bacterial vaccines being employed only in those instances where cultures cannot be obtained to prepare autogenous bacterial vaccines. The freshly isolated culture will be identical with the organisms present in the natural infection. There will be no antigenic differences in the autogenous preparation and which would or might prevail in a stock vaccine. In some cases there is considerable difficulty in the obtaining and even in the identifying of the specific infective agent or considerable time may be lost in preparing the autogenous bacterial vaccine which may be needed quickly, as in the treatment of acute infections. In such cases stock bacterial vaccines are employed until the autogenous bacterial vaccine is prepared. In certain instances (as in gonococcic vaccine, etc.), there is much time lost in preparing an autogenous bacterial vaccine even if the gonococcus can be obtained and it has been found that in such cases the stock bacterial vaccines already available possess but slightly inferior therapeutic value. In these cases the stock bacterial vaccines are more frequently used, rather than resort to the delay of preparing, if at all, an autogenous preparation.

The following stock bacterial vaccines (Dudgeon, "*Bacterial Vaccines and Their Position in Therapeutics*", 1927, London) are also advocated in the treatment of chronic and occasionally acute infections:

Pertussis or whooping cough vaccine is used by some in the treatment of whooping cough.

Pertussis vaccine mixed (containing in addition to *Bacillus* (*Hemophilus*) *pertussis* bacteria associated with the *H. pertussis* in infections of the respiratory tract as polyvalent *streptococci, staphylococci, and pneumococci, N. catarrhalis* and *H. influenzae*) is also used for this purpose.

Plague vaccine is employed occasionally experimentally in conjunction with antiplague serum in the treatment of bubonic or other types of plague.

Typhoid vaccine is used by some in the treatment of typhoid fever (Wherry and Associates, *J. Infect. Dis., XLIII,* 189).

Triple vaccine is used in the treatment of all conditions diagnosed as typhoid fever, and also used in non-specific vaccine therapy.

Other stock bacterial vaccines or bacterins used in the treatment of infections are:

Acne vaccine (Vaccinum Acne) is used in acute and chronic acne, caused by the acne bacillus (*Corynebacterium acnes*). *Acne vaccine mixed* or *combined* known also as Staphylo-Acne Vaccine (containing *Staphylococcus aureus, Staphylococcus albus* and *acne bacillus*) is used in acute and chronic (especially pustular types of) acne, caused by these organisms.

Brucella melitensis (Undulant fever) vaccine (consisting of *Brucella melitensis, var. abortus and var. suis*) is being employed in the treatment of undulant fever or Malta, Mediterranean or Rock fever. Worthwhile results have not been extensive. Intravenous therapy is advocated as most effective for

Brucella melitensis infections (*Policlinico,* Rome, March 23, 1935), (*Deut. med. Wochschr.,* 1934, vol. *60,* 788).

Brucella Vaccine or Bacterin is also employed as an aid in the diagnosis of brucellosis. The test is performed by injecting intradermally 1/50 cc. of a four billion dilution of the vaccine (containing per cc. 2 billion killed Brucella abortus (bovine) and 2 billion killed Brucella suis (porcine)). Observations are made after 48 hours. A positive reaction indicates infection with any of the species of Brucella. The sensitivity in man is such that response by positive skin tests is possible to all three varieties of Brucella, though the actual infection is caused by only one of the three species.

Catarrh ("cold") vaccine mixed or combined (consisting of killed respiratory bacteria and generally of *Neiserria* (*Micrococcus*) *catarrhalis, Streptococcus pyogenes, Staphylococcus aureus* and *albus, Diplococcus pneumoniae* (pneumococcus), *Klebsiella pneumoniae* (Freidlander bacillus), and (with or without) *Hemophilus* (Bacillus) *influenzae*) is used as a preventive and in the treatment of mixed infections of the respiratory mucous membranes, common colds, and catarrhal conditions of the naso-pharyngeal tract. For use either to increase resistance to or as a curative agent in the treatment of influenzal infections, the *H. influenzae* is always present, (*Annual Report,* 1931, *So. Afr. Inst. Med. Res.*). Many workers conclude that bacterial vaccine therapy does not appear to influence the incidence of colds (*J. Mich. Med. Soc.,* 1935, *XXXIV,* 157).

Oral vaccines containing organisms commonly found in respiratory infections are also available. *Respiratory Mixed Vacagen* an enteric-coated tablet containing the soluble antigenic substances of a mixed cold (respiratory) vaccine is marketed. The dosage recommended is a tablet daily one hour before breakfast (on an empty stomach) on seven successive days. Developed immunity may be maintained by taking one tablet once or twice each week.

Colon bacillus (Escherichia (Bacillus) coli) vaccine is used in infections caused by this organism after the acute symptoms have subsided, such as in cystitis, pyelitis, catarrhal jaundice, fistula-in-ano, etc. *B. Coli mixed bacterin* usually with polyvalent *streptococci, staphylococci, and pneumococci* is also marketed. *Van Cott's Vaccine* is a mixed B. coli vaccine.

Gonococcic (Neisserian or Urethritis) vaccine made from killed gonococci (*N. gonorrheae*) is used with but little success in infections caused by the gonococcus, as in acute and chronic gonorrhea, prostatitis, gonorrheal rheumatism, etc. *Mixed gonococcus vaccines* containing in addition other bacteria commonly associated with the gonococcus as *streptococci* (all types), *staphylococci, E. coli, pneumococci and diphtheroid bacilli* are employed in the treatment of complications as an aftermath of gonorrhea, (Harrison, "*Diagnosis and Treatment of Venereal Diseases*", 1931, London). Various antigenic preparations were considered at the symposium on treatment of gonorrhea at the 1934 American Medical Association meeting (*J. A. M. A.,* 1934, *103:*1819, 1821).

Pneumococcic vaccine (all three types of pneumococci), and *Pneumococcic vaccine mixed* (all types of *pneumococci, staphylococci and streptococci*) are recommended for immunization and are used with some success in the treatment of infections by these organisms (*Bull. N. Y. Acad. of Med.,* June, 1933).

Pyorrhea bacterin mixed is used by some in the treatment of pyorrhea alveolaris. It is prepared from strains of bacteria isolated from cases of pyorrhea and usually contains the following killed organisms: *streptococci* (all types), *pneumococci* (types I, II and III), *staphylococci* (*aureus* and *albus*), *Neisseria catarrhalis, Hemophilus influenzae* and *diphtheroid bacilli.*

Staphylococcic vaccine (all strains of the aureus and albus types and occasionally the citreus) is used in the production of immunity in minor infections caused by the staphylococcus and in the treatment of boils, carbuncles, acne and other chronic or recurrent types of infection caused by this organism.

Staphylococcic vaccine mixed (*staphylococcus* with one or all of the following: *streptococcus, pneumococcus* and *Escherichia* (*Bacillus*) *coli*) is used in mixed infections caused by these organisms, in rheumatic infections, and in non-specific vaccine therapy.

Streptococcus vaccine (prepared from all strains of streptococci) is used in the treatment of infections by this organism. Encouraging results have been reported in the use of such a bacterial vaccine in the treatment of chronic rheumatoid arthritis (*J. A. M. A.*, 1934, *103*:1357). At a conference sponsored by the American Committee for the Control of Rheumatism held in Milwaukee in June, 1933, there was considerable discussion on the use and value of streptococcic vaccines and antigens. Hency (*Proceedings of the Staff Meetings of the Mayo Clinic*, 1933, Vol. *VIII*, No. 38), reports of its efficiency and mentions the fact other workers expressed the belief that the value of vaccines in the treatment of arthritis has not been proved. On the other hand, the arthritis committee of the Brit. Med. Assoc. (*Brit. Med. J.*, 1933, I, 1044) presents favorable reports.

VACCINES NOT AVAILABLE FOR ALL DISEASES

● Theoretically it would appear possible to be able to create an active immunity by artificial means against all those diseases in which the microorganisms or causative agents are obtainable and capable of being used for the preparation of attenuated suspensions or vaccines. Practically, however, this has not been found of value. In many instances some of the bacterial vaccines which have been and can be prepared fail to stimulate the production of protective substances or antibodies. Others produce such a small amount of the latter as to afford a protection which will last but a short period of time, and such bacterial vaccines are, therefore, of little practical or commercial value. In other instances the results are doubtful. As we learn more about infective agents or develop more efficient techniques in preparing them for administration, and as the use of methods of administration and proper dosage which will suit each specific product are introduced, the list of agents to be used for creating an active immunity will be larger. Perhaps in some instances if we employ some preliminary medication or treatment before using the vaccine there will be developed a greater capacity for antibody production on the part of our body cells.

In all localized infections and in conditions previously mentioned antibodies may develop naturally, but in numbers which are not sufficient to overcome the infection. The injection of the bacterial vaccine causes the inactive healthy tissue cells to actively produce large numbers of antibodies which are carried (if possible) to the infected foci, so as to aid the infected tissues in overcoming the infection.

DOSAGE AND VALUE OF BACTERIAL VACCINES

● In all the above cases vaccine treatment is started with the subcutaneous, or by some intramuscular injection usually of small doses (approximately 50,000,000, depending upon the organism) (less for children). This dose is gradually increased, inoculations being made at three-to-eight day intervals, depending upon the reaction received and clinical results obtained. Dosage and frequency of injections are to be regulated to suit each individual case. Clinical evidence has revealed that children tolerate bacterial vaccines better than do adults. It is therefore a frequent occurrence to observe that the average dose recommended is given to normal individuals regardless of age or weight. More recently it has also been found that intradermal injections, while producing the greater reaction, are nevertheless more productive of antibody response as well as more rapid than other methods of administration (Besredka: *Local Immunization,* 1927, London). Lanolin and other non-specific substances are being advocated as vector agents when administering bacterial vaccines and other antigens. They act by retarding absorption as the antigens stimulate the defenses of the animal (Pinoy, 1935, *Comp. rend. soc. biol., 118:*442).

Mention was made previously of the use of vaccines by oral administration. Attempts have been made to use them by instilling the vaccine into the nasal passages (see under Whooping Cough Vaccine and *Science News Letter, 34,* 285 (1938), where in the latter there is described treatment of sinus disease by local instillation of antigens). Rectal administration has been reported. Certain vaccines are administered intravenously, especially in fever (non-specific) therapy.

Perhaps the most striking way of setting forth the value of bacterial vaccines in prophylaxis is to mention data concerning typhoid prophylaxis. Had the rates of the Spanish American War been in effect at the period of the World War, the United States would not have had 1572 cases of typhoid fever among its soldiers, but more than 500,000 cases.

While there is indisputable evidence as to the value of stock bacterial vaccines in prophylaxis, the evidence as to their value therapeutically is not so marked and only comparatively in few instances. The whole subject of bacterial vaccine therapy in the treatment of disease must await further experimental and clinical observations before an exact evaluation can be forthcoming.

The value of bacterial vaccine treatment in acute infections, especially acute systemic infections, is questionable. The proposed method of treatment is first to have prepared an autogenous bacterial vaccine wherever possible. Until this is made, stock bacterial vaccines are to be used. Large doses are advised to be given daily (usually intravenously) until symptoms subside, and then at two or three-day intervals, until recovery is complete. (See also non-specific protein therapy).

A consideration of the bacterial vaccines which have proved most efficient in practice follows. Others have been considered previously.

XIV

VACCINUM TYPHOSUM

Typhoid Vaccine

Bacterial Vaccine Made From the Typhoid Bacillus

Vac. Typhos.—Typhoid Prophylactic, Enteric Vaccine

"BACTERIAL Vaccine made from the Typhoid Bacillus is a sterile suspension of killed typhoid bacilli (*Eberthella typhi*) in physiological solution of sodium chloride or other suitable diluent. The Vaccine shall contain, in each cc., at least 1,000,000,000 typhoid organisms.

Description and Physical Properties

● A more or less turbid, whitish liquid, nearly odorless, or having a faint odor due to the presence of a preservative. Bacterial Vaccine made from the Typhoid Bacillus must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol if either of these is used).

Bacterial Vaccine made from the Typhoid Bacillus must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Bacterial Vaccine made from the Typhoid Bacillus,* and indicate the number of organisms represented in 1 cc., the name, address, and license number of the manufacturer, and the date beyond which the Vaccine may not be expected to retain the potency prescribed by governmental authority.

Storage

● Preserve Bacterial Vaccine made from the Typhoid Bacillus at a temperature between 2° and 10° C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● One-half cc. (500,000,000 organisms) and 1 cc. (1,000,000,000 organisms), the latter dose to be repeated once."

PREPARATION

● In the preparation of typhoid vaccine, the selection of the strain of *Eberthella typhi* is important. The "Rawlings" strain of typhoid obtained originally from Wright in England and isolated from a fatal case in 1905

during the Boer War is most frequently used. Eighteen to twenty-four hour old beef-infusion agar cultures are washed off with and the growth is suspended in sterile saline (and after agitating well by hand or preferably in a mechanical shaker), the number of bacilli per cc. are determined.

The suspension is then heated at 53°C. for one hour, tricresol, phenol or other equally effective antiseptic is added to a 0.3% or suitable concentration, and tricresolated (0.3%) salt solution is added to produce the desired bacterial content (generally one billion bacteria per cc.). Sterility and toxicity tests are conducted by making aerobic and anaerobic cultures and inoculating a laboratory animal (usually a guinea-pig but a rabbit or a mouse or any two of these may be used). If contamination is revealed, the material is discarded. Marketable preparations are generally tested for potency by inoculating several (a series of three) rabbits and then determining the agglutination reaction (agglutinin content or titer) of their sera with the living culture of *E. typhi* as a measure of the immunity response induced. A serum prepared under identical conditions by immunizing rabbits against a standard vaccine obtained from the National Institute of Health is included in the agglutination tests for purposes of comparison. The average titer of the sera produced should approximate, equal or exceed that of the control serum. The expiration date for this as for all other bacterial vaccines is 18 months after date of manufacture or date of issue.

Strains of E. Typhi Used

● The use of a single avirulent strain as prepared by Russell (*J. A. M. A.*, *73*, 1863 (1919)) and as used in producing the Army vaccine has been and is being questioned by workers even though such culture has proven to be capable of producing large quantities of immune bodies. As a general rule, bacterial vaccines serve best if they are polyvalent. Experiments by Hooker (*J. Immunol.*, *11*, 1 (1916)) and other workers have revealed antigenic differences among strains of ***Eberthella typhi.*** More recently, experimental studies by Grinnel (*J. Immunol.*, *19*, 457 (1930); *J. Exptl. Med.*, *54*, 577 (1931); *56*, 907 (1932)), have revealed that it may be possible to improve the efficacy of typhoid vaccine by using the smooth strain of *E. typhi.* He showed that a vaccine prepared from a smooth virulent strain (S-type of *E. typhi*) was more potent and more effective in its immunizing power than one prepared from cultures of *E. typhi* (rough or R-type). Grinnel pointed out that most of the cultures of the Rawlings strain used for the production of marketable typhoid vaccine were in the rough phase of dissociation. He further mentions that a high agglutinin content is not as safe an index of immunization against typhoid fever as is the measure of yield of specific bactericidal substances. The latter are produced by the smooth strains which alone contain a carbohydrate fraction, which he claims is the essential antigenic fraction and which takes little or no part in the agglutination reaction. Though the Rawlings strains of *E. typhi* are universally used in the preparation of typhoid vaccine, a carrier strain of *E. typhi* designated No. 58 appears to be a more suitable strain and as one of substantial antigenic value will probably replace the older strain (*Publ. Health Rep.*, *52*, 829 (1937)).

An editorial in the Canada Public Health Journal (*29*, 511 (1938))

suggested that the typing of typhoid bacilli by the technique of Craigie and Yen (*Can. Publ. Health J.*, *29*, 284 (1938)) may present a new approach to the problem of obtaining a preventive vaccine against typhoid fever. This may not only be of greater value in prophylaxis but there may also be available types which will result in important advances in the serum therapy of typhoid fever.

In Hygienic Laboratory Bulletin No. 110 there is given the procedure for making and testing typhoid vaccine by a technique, which is a modification of the method employed at the United States Army Medical School (Bulletin 2, War Department, Office Surgeon General, p. 33). The details of this technique follow.

DIRECTIONS FOR MAKING TYPHOID PROPHYLACTIC

Culture

"1. Plate the Rawling culture on at least one set of Endo plates; incubate 24 hours.
2. Examine colonies and mark at least three which have typical appearance and are large and well separated.
3. From each colony make a transfer to three tubes. (1) Two broth tubes; (2) a stab and slant of Russell's double sugar agar. Keep corresponding tubes together; incubate twenty-four hours.
4. Examine growth and make macroscopic agglutinations by adding one drop of antityphoid serum of high titre to one broth tube inoculated from each colony. Incubate one-half hour at 37°C.
5. Make several agar slants from the broth tube corresponding to the one giving best positive agglutination and typical appearance on Russell's medium. Incubate 24 hours at 37°C.
6. Add three-fourths cc. of salt solution to each slant; scrape off growth, and collect the emulsion in large test tubes or Erlenmeyer flasks.
7. From each tube make a heavy smear and stain by the Gram method to determine purity of emulsion.

Media, Etc.

1. Make 3 per cent agar, +0.5, to phenolphthalein.
2. Sterilize 10 to 30 one-liter bottles with flat sides, plugged with absorbent cotton, by dry heat at 170°C. for 1½ hours.
3. Put 100 cc. agar in each bottle, and sterilize in autoclave at 15 pounds for one hour.
4. Let agar harden over night with bottles flat. Invert and place in incubator on slight slant for 24 to 48 hours.
5. Make 0.85 per cent NaCl solution; autoclave at 15 pounds, 1½ hours. Plant 5 cc. of the salt solution from each flask in freshly heated and cooled glucose broth fermentation tubes. Incubate 48 hours; discard if contaminated.
6. Make several absorbent-cotton swabs on wire rods; put in test tubes and sterilize by dry heat at 170°C. for 2 hours.

Vaccine

1. Inspect each bottle for evidence of contamination, flame plug, and remove plug and flame mouth of bottle. Remove water of condensation with a sterile pipette.
2. Inoculate each bottle twice with well-charged swab from a tube of emulsion. Invert on slant and incubate 24 hours. Make plate cultures

from emulsion which is left. (Inoculation may be done by placing a thin layer of the emulsion upon the agar and flowing it evenly over the surface.)

3. Examine each bottle with great care and discard any bottles which have growths not typical of a typhoid culture or which show Gram-positive organisms on staining. This is a most important step in the procedure, because if any spore-bearing organisms are passed over and get into the vaccine they will not be killed by subsequent measures and the vaccine will not be sterile.
4. Add 30 to 50 cc. of salt solution to each bottle; place on slight slant, so that salt solution will moisten growth for few minutes. Wash off growth by holding the flask in the hands and slightly tilting the bottle from side to side so that the fluid flows across the surface of the agar. Collect the emulsion in 500 cc. flasks.
5. Mix collections, measure emulsion, and take sample for counting.
6. Pour emulsion into 500 cc. flasks, put tin foil on plug, weight with lead around neck and put in water bath. A control flask with salt solution and thermometer is put in bath. Another thermometer is suspended in the bath direct. The water should come above the fluid in the flasks by 1 to 2 inches.
7. Heat emulsions at 53°C. to 54°C. in well-agitated water bath, watched constantly to keep the temperature accurate. Remove flask after one hour at this temperature.
8. The neck of the flask should be thoroughly flamed to a point below the level heated by the water bath.
9. From the count estimate amount of salt solution to be used to give 1,000,000,000 bacilli per cubic centimeter and add trikresol to diluting fluid; so that the final strength of trikresol will be 0.4 per cent. Dilute vaccine according to count in the large sterile bottles. Trikresol should be entirely in solution before adding to vaccine. Its solution is more prompt if added to warm salt solution and shaken during the addition.
10. After 12 to 24 hours at room temperature take sample for culture.

Counting

1. A well-mixed sample of the emulsion is placed in a small sterile conical glass.
2. A portion of this is diluted in a red-blood cell counting pipette using the following solution as a diluent: five per cent phenol solution, 100 cc.; Ziehl-Neelsen carbol-fuchsin, 0.25 cc. Mix and let stand 10 minutes and filter.
   This diluting fluid kills the typhoid bacilli and stains them light red.
3. Count the number of bacteria in a Helber counting chamber in a manner similar to counting blood cells.
4. Estimate the number of bacteria per cubic millimeter of emulsion. (The Helber counting chamber is 0.02 mm. deep, the blood counting chamber 0.1 mm. Hence the capacity of the Helber chamber is one-fifth that of the blood counting chamber.)

Tests

1. Plate two loopfuls of emulsion left from inoculation on 2 sets of plates.
2. Plate 0.1 cc. heated vaccine in each of 3 plates. These plates should show some colonies of typhoid but no others.
3. Plant cresolized vaccine in at least 6 anaerobic tubes, of freshly heated

and cooled glucose broth, 5 drops in 3 tubes and 20 drops in 3 others. These tubes should be sterile. If not, the vaccine must be discarded. Incubate 7 days.

4. If above tests are satisfactory at end of 48 hours the following tests are proceeded with: Inoculate 2 guinea pigs, 1.5 cc. intraperitoneally; 2 white mice, 0.5 cc. subcutaneously. Give each of 3 rabbits, 0.5 cc. after 3 days 1 cc.; after 6 days 1 cc., all subcutaneously. Agglutination is tested after 5 days and should equal the controls.

Final agglutination should run at least 1-500 dilution. The mice and the guinea pigs are used as a test for tetanus. The mice also serve as a check on the amount of preservative. The rabbits are used as a test for agglutinin production.

Filling of Ampuls

1. Any apparatus that reduces the possibility of contamination to a minimum may be used.
2. Care must be taken to see that cracked ampuls are excluded and that the seal is perfect. To do this, place the filled ampuls in a water-bath at 40°C.; stir gently to insure uniform heating to this temperature.

Remove the ampuls to an iced water bath to which sufficient methylene blue is added to give it a deep blue color. After sufficient time has elapsed to allow all ampuls to be cooled, remove the ampuls and rinse in tap water. The contents of defective ampuls will be colored blue.

Test of Finished Product

1. At least 3 ampuls, selected at random from each lot of the finished product are examined, or 3 per cent if more than 100 are filled at one time, up to 20 ampuls for each lot. These are tested for sterility by planting from each ampule 5 drops in a Smith fermentation tube, and 20 drops in a similar tube, containing about 30 cc. of recently heated and cooled glucose broth having a reaction of +0.5 per cent to phenolphthalein at 100°C.
2. The tubes are inoculated and examined after two, four and seven days; they should be agitated after each examination and the lowest part of the bend should be examined for slight growth before agitation.
3. If growth is found in any tube, a second test is made, using double the number of ampuls used in the first test.
4. If the second test shows growth, the vaccine is discarded."

ARMY MEDICAL SCHOOL TECHNIQUE OF PREPARING TYPHOID VACCINE

● The following procedure is employed at the United States Army Medical School for the production of the typhoid vaccine for use in the U. S. Army and for other Government services. For more complete details, see *U. S. Publ. Health Reports, 52,* 829 (June 25, 1937).

THE PREPARATION SUBDIVISION

"*Glassware*—As glassware comes in at the receiving entrance it is sterilized by steam under pressure, by boiling, or by a combination of both methods. It is then washed, drained, and finally dried in a large oven drier designed for the purpose. The small ampules, vials, and bottles which are filled with the vaccine for distribution are washed on a manifold water-jet device which permits the simultaneous rinsing of 150 of these small containers. After being thoroughly cleansed, the water remaining in them is blown out by

compressed air. They are then placed in metal boxes and sterilized by hot air for 5 hours at 240°C. In these boxes they are taken to the filling cubicle and there receive their specified amounts of the finished vaccine.

Culture media—Veal infusion, the basic ingredient of the culture media used in the preparation of the vaccine, is made as follows:

Lean veal (freed from fat and fibrous tissue)	10,000 gm.
Distilled water	10,000 cc.

Place in the refrigerator for 18 to 24 hours. Remove and bring slowly to the boiling point; continue boiling for 45 minutes; strain through cheesecloth and press out as much fluid as possible; bring the volume up to 10,000 cc. by addition of distilled water; place in 2-liter flasks; autoclave at 15 pounds for 30 minutes; store in refrigerator. Stock infusion more than 21 days old is not used in the preparation of vaccine.

Veal infusion broth

Peptone	10 gm.
Sodium chloride	5 gm.
Agar (powder)	18 gm.
Veal infusion	1,000 cc.

Mix and heat to dissolve the agar; adjust the reaction to pH 7.4; fill into plugged and sterilized Kolle flasks (each flask receives 45 cc.); autoclave at 15 pounds for 30 minutes; check pH (if it is not about pH 7.2 it is not used for vaccine production).

Upon removal from the autoclave, place the Kolle flasks on a level table to allow the agar to harden; incubate them for 24 hours to check their sterility; store at room temperature until they are to be inoculated. For convenience, the flasks are handled in metal trays each holding 50 flasks. In the parlance of the laboratory such a tray of 50 flasks is a unit designated as a "section".

A few hours before they are to be inoculated, the sections are placed in a preheating incubator (held at 45°C.) located just outside the inoculating cubicle. This is to insure their being near blood heat when they are planted, to eliminate the lag in growth which results from chilling.

Sterility broth—This medium, recommended by the National Institute of Health, United States Public Health Service, for use in routine sterility tests, is prepared as follows:

"To 8 kilograms of ground fresh meat freed from fat, 16 liters of distilled water are added and the mixture is infused in the ice chest 24 hours. Sixteen liters of juice are squeezed out through cheesecloth, heated in streaming steam for 1 hour, autoclaved at 15 pounds pressure for 30 minutes, filtered through moistened paper, and brought up to the original volume. Five grams of sodium chloride per liter and 10 grams of peptone per liter are added and the broth is stirred until solution takes place. The pH is adjusted to such a point as experience shows will result in a pH of 7.5 in the final broth in the fermentation tubes by adding solution of sodium hydroxide. The broth is heated in streaming steam for 30 minutes, filtered through moistened paper, placed in glass-capped Smith fermentation tubes, each containing at least 25 cc. and holding a seal of a least 1 centimeter in the open arm, and autoclaved at 15 pounds pressure for 20 minutes. The Smith fermentation tubes should be in racks which facilitate tipping oxygen bubbles out of the long arm when hot, or which allow such bubbles to flow out of the tubes while the heating is going on, and which permit ready inspection of all parts of the tube for growth. The design in use

at the National Institute of Health is recommended. If the broth is not to be used immediately, it may be filtered into flasks containing not more than 1,500 cc. each, sterilized by streaming steam for 2 hours or by autoclaving for 20 minutes at 15 pounds pressure, and stored prior to filling into fermentation tubes.

"To detect contamination, the fermentation tubes may be incubated for a few days, or an adequate number (about 20 per cent) of controls planted with material known to be sterile, simultaneously with the tests. Not more than 5 hours before planting, the fermentation tubes shall be heated to fully 100° C. for 30 minutes and immediately tipped to expel the air from the long arm unless the tubes are arranged in the sterilizer so that the bubbles leave each tube while being heated. The pH at this point should be between 7.2 and 7.8; but instead of repeated adjustments of reaction during the process of preparation of the medium, it is preferable to add enough alkali in the beginning to insure a proper reaction when all the steps are completed. The amount to be added can be ascertained only by repeated trials, using the same ingredients. No acid should be added at any point in the process.

"No dextrose need be added to the batch of medium provided a preliminary test has shown that it contains an appreciable amount of muscle sugar. This test is made by inoculating two Smith fermentation tubes, filled with the fully prepared and sterilized medium, with an active strain of colon bacillus. If, after overnight incubation, both fermentation tubes show a bubble of gas filling the tip of the closed arm, sufficient sugar may be assumed to be present. If not, or in the absence of such a test, approximately 0.03 per cent of dextrose should be added just before the final heating in the fermentation tubes. For this purpose 1 per cent dextrose solution in flasks containing not more than 50 cc. each should be sterilized in the autoclave at 15 pounds pressure for 15 minutes, and added to the broth in the proportion of 1 cc. to each fermentation tube. Planting is done within 5 hours after the broth has cooled."

Buffered saline solution—This is the menstruum in which the agar growth of typhoid bacilli is suspended, 30 liters being provided for each "section" of 50 Kolle flasks. For convenience this is distributed into four 8-liter bottles, each containing 5 liters of the saline, and the remainder in 1-liter and 2-liter Erlenmeyer flasks.

The formula for the buffer solution is as follows:

NaH_2PO_4	28.81 gm.
Na_2HPO_4	125.00 gm.
Distilled water, q. s. ad	1,000.00 cc.

The buffer solution is added to the saline (which is 0.85 per cent NaCl in distilled water) in the proportion of 20 cc. of buffer to each liter of saline solution. The buffered saline solution is then sterilized at 15 pounds for 1 hour.

Mucin—For the preparation of the mixture of crude hog stomach mucin in which the test doses of living typhoid bacilli are suspended, for injection intraperitoneally into mice, three sterile solutions are used at the present time:

1. To 75 gm. of mucin, powdered by grinding in a ball mill, add 800 cc. of distilled water, mix thoroughly and allow to stand in the refrigerator overnight (granular mucin requires 18 hours in the refrigerator). Remove and stir with a motor-driven mixer for 2 hours; make up the volume to 1320 cc. with distilled water; autoclave at 10 pounds for 15 minutes.

2.

K_2HPO_4	20.25 gm.
KH_2PO_4	4.32 gm.
Distilled water q. s. ad	165.00 cc.

Mix and autoclave at 15 pounds for 30 minutes.

3.

Dextrose c. p.	7.5 gm.
Distilled water q. s. ad	15.0 cc.

Sterilize by filtration or by heating at 80°C. for 1 hour on each of 3 successive days.

Mix these three solutions under sterile conditions, using flame technique. Check the reaction—it will be pH 7.2; test for sterility. Store the mixture in a refrigerator. Before a portion is withdrawn for use, agitate the mixture thoroughly until all sediment is uniformly in suspension.

In order to secure information concerning some of the essential qualities of a useful mucin mixture, estimations are made, on every lot, of viscosity and total and nonprotein nitrogen content. Furthermore, active studies are in progress which have as their aim a simplified process and a uniform product as well as the elucidation of the problem of the mode of action of the mucin.

Cotton swabs—These are ordinary cotton swabs, of rather large size used for inoculating the agar in the Kolle flasks. They are made by applying absorbent cotton to the ends of iron wire rods, and are inserted into large test tubes and sterilized in the autoclave for 1 hour at 15 pounds pressure.

Collecting flasks—These are heavy-walled Erlenmeyer flasks with a graduation mark indicating 2,000 cc. They are fitted with two-holed rubber stoppers. One of the openings in the stopper carries a glass tube 3½ inches long which, in operation, is attached by sterile rubber tubing to the vacuum system; the other opening is for a shorter glass tube to be connected with the harvesting tool. This latter tube is flanged at its lower end and has attached to it a filter bag consising of three layers of gauze. The collecting flasks, with their stoppers in place and covered with muslin, are autoclaved at 15 pounds for 30 minutes.

The harvester— The harvester is a metal tube 13¼ inches long with a short section 1½ inches long fixed to its distal end to form a T. The transverse piece will pass into the Kolle flasks through the broad oval neck; it is closed at both ends and has a narrow opening or slot running nearly its entire length on one flattened side. The harvester serves as a rake to loosen the growth of bacteria from the surface of the agar, then the suspension thus formed is aspirated, through the slot, into the collecting flask. The harvesters, after being wrapped in muslin, are sterilized in the autoclave at 15 pounds for 30 minutes.

Cotton stoppers—These are gauze-covered cotton plugs provided to replace the rubber stoppers in the 2-liter collecting flasks. They are wrapped in muslin and sterilized in the autoclave at 15 pounds for 30 minutes.

Graduated cylinders—Graduated cylinders of 1,000 cc capacity are

stoppered with gauze-covered cotton plugs, and, with muslin tied over their stoppers, they are sterilized in the autoclave at 15 pounds for 30 minutes.

Pipettes—Pipettes of 10 and 25 cc capacity are sterilized by hot air at 170° C. for 2 hours.

The bottling apparatus—This is assembled, wrapped, and sterilized in the autoclave at 15 pounds for 30 minutes.

The bottling cabinets—The bottling cabinets are wrapped and sterilized in the autoclave at 15 pounds for 60 minutes.

Vaccine ampules and vials—Vaccine ampules (1 cc) and vials (5, 10, 25, and 50 cc) are washed and dried, packed in trays, in metal boxes, and sterilized by hot air at 240° C. for 5 hours.

Rubber stoppers—Rubber stoppers to fit the vaccine vials are of special composition. They are washed thoroughly in several changes of hot water, then autoclaved for 30 minutes at 15 pounds pressure. Then they are washed again, placed in 0.8 percent phenol solution, and again autoclaved while still immersed in the phenol solution at 15 pounds for 30 minutes. For use they are removed from the phenol solution in small quantities as needed to stopper the filled vaccine bottles.

Rubber gloves—Rubber gloves are washed in 2 percent phenol and sterilized at 15 pounds pressure for 30 minutes.

Gowns and other clothing—Gowns and other clothing worn exclusively by the technicians while working in the cubicles are sterilized in the autoclave at 15 pounds for 30 minutes. The sterile clothing is put on after the technicians have entered the cubicles.

Shoes—Shoes are wet just before use by dipping their soles in a shallow pan containing a gauze pad wet with cresol solution.

Large earthenware jars—Large earthenware jars containing 3 percent cresol solution are provided for the disposal of tubes and other materials contaminated by living bacteria.

The seed culture—Since October 12, 1936, *Eberthella typhi* 58 has been used in the preparation of the Army typhoid vaccine. This strain was isolated from the feces of a chronic carrier, in Panama, who had typhoid fever in 1913 and who, since that time, has been under the continuous observation of Dr. L. B. Bates, Director of the Board of Health Laboratory, Panama Canal. The culture was received in September 1934, immediately after isolation from the carrier.

The surface colonies of this strain, on plain agar plates, are relatively large, with a moderate dome which is somewhat flattened; edges are slightly undulate; surface is smooth; consistency is moist and homogenous; the growth mixes evenly with saline; it does not agglutinate in 6.4 percent saline; it grows with uniform turbidity in broth without granular clumps; surface pellicle is absent in young cultures, and there is no sedimentation. The bacilli are actively motile, and are uniformly small, short rods. Studies of the bacterial count of finished vaccines seem to indicate that Strain 58 has more tendency to autolyze than does the Rawlings strain. When suspended in mucin their virulence is such, when inoculated intraperitoneally, that 10, 100, or 1,000 living bacilli will kill all white Swiss mice or black mice (Strain C-57) of 16 to 18 gm. weight within 72 hours.

It is well known that many bacteria lose certain parasitic attributes after continuous cultivation on artificial culture media. In order to avoid

such changes and to maintain the vaccine cultures without alteration or dissociation, large numbers of ampules containing the frozen and dried cultures are kept in stock. For this method of preservation the apparatus of Flosdorf and Mudd is used. This apparatus consists of a glass or metal manifold having 24 outlets with a main and secondary condenser connected in series, immersed in a bath of dry ice (solid CO_2) and an antifreeze solution, contained in an insulated vessel. The secondary condenser is connected with the vacuum pump.

Broth suspensions of the agar cultures, grown for 12 hours at 37.5° C., are distributed in small amounts, usually 0.2 cc, into ampules. The ampules are immersed in a dry-ice bath (temperature about –78° C.) for 10 to 15 minutes. At the end of this time the ampules are rapidly connected to the manifold and the vacuum pump is started. Moisture is removed from the frozen material by sublimation *in vacuo* and is trapped in the condensers. A vacuum of 0.70 mm Hg, or less, will keep the cultures frozen until drying is complete. This requires about 6 hours. Then, with the vacuum pump still operating, the ampules are sealed off individually, using a gas-oxygen hand torch. The dried cultures are stored at 2° to 5°C.

When a lot of vaccine is to be manufactured, an ampule is broken, about 0.2 cc sterile distilled water is added, and a culture in veal infusion broth is made from the resultant suspension. After incubation for 2 hours, streak cultures are made on veal infusion agar in Petri dishes; a series of 10 plates is made; these cultures are grown in the incubator overnight. The following morning the colonies developed are studied with great care, using a binocular dissecting microscope. Typical smooth colonies are fished to agar slants in large (50 by 200 mm.) test tubes; a part of the same colony is planted to a tube of Russell's double sugar agar, by streak and stab. These cultures are all incubated overnight. The growth on each of the large agar slants is the seed for the inoculation of one section of 50 Kolle flasks. The growth in these large culture tubes is suspended in 25 cc. of veal infusion broth and the suspension transferred to clean sterile tubes of the same size. After incubation for 2 hours these tubes are ready to be taken to the cubicles and used for the inoculation of the agar in the Kolle flasks.

The few drops of suspension left in the culture tubes are studied to determine the purity and identity of the growth. Motility is checked in hanging drop, and staining reaction and morphology are ascertained on a Gram-stained slide. A suspension showing any tendency to auto-agglutination is discarded; those in which there are long thread-like forms are not used, partly for the reason that the threads interfere with accuracy in counting.

The Russell double sugar cultures, which were made as duplicates from colonies used to inoculate the seed cultures, must show typical acid butt and alkaline slant, with no gas formation. The growth from these Russell slants is suspended in buffered saline and used to make agglutination tests. The result must be positive to the limit of potency of the agglutinating serum used. The tubes containing the appropriate mixtures of agglutinating serum and suspension are held at 56°C. for 2 hours and then placed in the refrigerator overnight.

The Production Subdivision

Planting the Kolle flasks—Before they are placed in the special incubator, where they are brought up to 45°C. preparatory to inoculation, the Kolle

flasks are examined for cracks and other flaws, for imperfect cotton stoppers, and for any indication of contamination of the agar. Only flasks perfect for the purpose are transferred from the preparation subdivision to the production subdivision.

Two technicians work together in the planting cubicle. They wear only sterilized clothing and only they enter the cubicle. The cubicle has been steamed and now is receiving sufficient sterilized air to create distinct positive pressure so that no drafts can enter through any accidental leak that may have escaped careful inspection.

The technique of inoculation is simple. The equipment used consists of the section of flasks, the tube of seed culture held in its support, the cotton swab, and the Bunsen burner. A flask is picked up, the cotton plug removed, the mouth of the flask flamed, the cotton swab inserted into the seed culture suspension, then removed and passed over all parts of the surface of the agar in the flask, the swab is replaced in the seed tube, the mouth of the Kolle flask is flamed again, the cotton stopper is put back into the flask, the flask is returned to its rack, which is inclined so that water of condensation will run off the surface and collect at the low edge of the agar. Then, flask after flask is inoculated in the same manner. When all the flasks of a section have been thus planted, the tray with its 50 flasks is placed in the specially constructed incubator, with temperature and humidity control and circulating air, where it remains overnight, usually from 18 to 22 hours at 37.5°C.

As an important check on the maintenance of purity of the seed culture suspension, throughout the process of planting the flasks the portion of the suspension remaining in the tube, at the end, is plated on plain veal infusion agar. This must be a pure culture of the typhoid organism.

Harvesting—Before the collection of the growth is begun, each Kolle flask, planted the day before, is inspected with the greatest care. Any flask showing the slightest suspicious evidence of atypical growth or contamination is discarded.

The materials required are the harvesting tool, flasks of sterile buffered saline, the 2-liter collecting flask with its rubber stopper and tubes, and the Bunsen burner. As noted in a preceding paragraph, one of the glass tubes has a gauze filter bag secured about its opening inside the collecting flask, the outer end of this tube being connected by means of sterile rubber tubing with the harvesting tool; the other glass tube is connected with the vacuum system. The rubber tubing for making these connections is sterilized separately in the autoclave, and the connections are made immediately before the harvesting begins; flame technique is used in this procedure.

Two technicians work together. A Kolle flask containing the growth of organisms is picked up by one man, who removes the cotton plug, flames the mouth, and pours into it about 20 cc. of buffered saline. It is passed to the other man who, first using the collecting tool as a rake, carefully scrapes the bacterial growth from the surface of the agar. The growth readily mixes with the saline and makes a heavy, milky suspension; this suspension is then aspirated into the flask by releasing, to exactly the proper degree, the pressure he maintains on the rubber tubing. The aspiration of air increases the danger of contamination. By the time the growth has been removed from the first flask, a second is ready with its 20 cc. of saline added. This procedure is continued until the suspended bacteria of the entire section are in the collecting flask. The rubber tubes

are removed from their glass connections in the rubber stopper, and the rubber stopper is taken out and replaced by a sterile gauze covered cotton stopper; then sufficient sterile buffered saline is added to bring the amount in the collecting flask up to 2 liters.

After thorough agitation to insure uniformity of the suspension, samples are removed—one of 10 cc. for counting, and one of exactly 2 cc. for the virulence test. The 2-cc. quantities from each of the collecting flasks, resulting from 1 day's work, are pooled and kept cold until diluted for the mouse injections—about one-half hour. After it is labelled, the collecting flask is carried to the killing cubicle.

Killing—The water bath provided for this purpose is heated by electricity, the temperature being automatically controlled to 0.1°C. The water is kept in constant circulation by a motor-driven pump to assure uniform temperature in all parts of the tank. It is of such size that 12 flasks of 2-liter capacity may be heated simultaneously. One flask containing water, and with a thermometer in it, serves as a guide to the rapidity with which the temperature in the other flasks rises and indicates when it reaches the desired maximum point (56°C.). The flasks rest on a perforated shelf raised from the bottom; the water is of such depth that its level is well above that of the suspension in the flasks. The flasks containing the concentrated suspensions are held in the water bath for 1 hour after the temperature in the control flask has reached 56°C. Upon removal from the bath, the flasks are allowed to cool to room temperature.

Standardization—While the suspension is in the water bath, the 10-cc. sample, collected at the completion of its harvesting, is counted. The technique employed is the direct method, using a Helber blood counting cell. The following are the steps in this procedure:

The heavy suspension received from the collecting flask is diluted with sterile buffered saline, in the proportion of 1 cc. of suspension to 29 cc. saline (in practice, 1 + 9; then, of this, 1 + 2); then this diluted suspension is mixed for counting as follows:

	cc.
Diluted suspension	1.0
Sterile buffered saline containing 1.0 percent formalin	3.5
Carbol-methyl violet solution[1]	0.5

The final dilution as counted is thus 1 to 150.

Place the mixture in a test tube; warm it over a Bunsen flame until it is almost at the boiling point; let it stand for 1 minute, then cool rapidly by placing it in ice water. With a capillary pipette, transfer a drop of the well-shaken mixture to a Helber cell-counting chamber, using a Hausser cover glass, 0.18 mm thick. On the microscope stage, find the ruled squares with the 2/3-inch objective; place a drop of cedar oil on the cover glass; turn to the oil immersion lens and bring the ruled area into focus; wait 10 minutes to allow the bacilli to settle into the same focal plane; count the number of bacteria in 20 or more, small squares, using the fine adjustment to detect bacilli which may not have settled; calculate the average number of bacteria per square. The squares are 1/20 mm by 1/20 mm; the chamber is 1/50 mm deep; therefore, if the average number of bacilli per square was found, let us say, to be five, we would have in each cubic millimeter of the suspension which was mixed with the stain: 20 × 20 ×

	cc.
[1] Saturated alcoholic solution methyl violet 6B	1.0
5 percent. aqueous solution of phenol, q. s. ad	100.0

50 × 5 = 100,000. The number per cubic centimeter would be 1,000 times this, or 100,000,000. This figure, finally, is multiplied by the number of times (150) the original suspension was diluted for counting.

(Note.—The complete mathematical problem involved herein is avoided by a practical "short-cut" formula, as follows: Count the total number of bacilli found in 20 squares; multiply this figure by 0.3; the result will be the number of liters of vaccine of 1,000 million per cubic centimeter strength to be made from the 2 liters of heavy suspension in a collecting flask.)

Virulence test—Black mice of a pure strain, known as Strain C-57,[1] have been found suitable for this test because of their relatively uniform susceptibility to typhoid infection by the intraperitoneal route. At the time of injection they weigh between 16 and 18 grams.

As previously noted, from each of the 2-liter flasks of suspension harvested on one day, 2 cc. are removed and mixed. This representative pooled suspension of live organisms is counted, in the regular way, then it is diluted so that the doses injected into the mice will be contained in 0.5 cc. Ringer's solution is used first to bring the number of bacilli in 1 cc. down to 10 times the strength of the suspension injected into the mice; the final (1:10) dilution is made in mucin. Thus, the standard doses used to test the virulence of Strain 58 are 10,000, 1,000, and 100 bacilli; these numbers are to be contained in 0.5 cc. Therefore, with Ringer's solution the original suspension is diluted first to 200,000 bacilli per cubic centimeter; of this concentration, 1 cc. is mixed with 9 cc. Ringer's solution, giving 20,000 bacilli per cubic centimeter; then 1 cc. of this second suspension is diluted with 9 cc. Ringer's solution, making a suspension of which 1 cc. contains 2,000 bacilli. Each of these three suspensions is then mixed in the proportion of 1 cc. plus 9 cc. of sterile buffered mucin mixture; the resultant mucin suspensions will contain in 0.5 cc. amounts, the proper doses for the mice—10,000, 1,000, and 100 bacilli.

For the purposes of this test, 10 mice are injected with each dose. The mice are held under observation for 72 hours, when the result is recorded. It is expected that all mice receiving the higher doses will die within this period; some of those receiving the lowest dose may survive.

As a check on the cause of death, at least two mice are examined and cultures are made from the heart blood on veal infusion and EMB agar plates. Colonies are fished to Russell's double sugar and subjected to microscopic examination and agglutination tests.

These tests for mouse virulence are believed to be important. The indications which may be drawn from work done at the Army Medical School are that virulence and immunogenic potency tend to parallel one another.

Dilution.—Harvesting is completed in the earlier hours of the morning. Then the collected bacilli are killed in the water bath while the numbers of organisms per cubic centimeter in each flask of suspension are being determined by direct count. These procedures accomplished, the work for the afternoon is that of diluting the heavy suspension with buffered saline so that each cubic centimeter will contain 1,000 million bacilli. As a preservative, and to kill any bacilli surviving the temperature of the water bath, tricresol is added to a concentration of 0.25 percent.

The materials placed in the cubicle for making the dilutions include the following: The 2-liter flasks of concentrated, heated, suspension of bacilli labeled to show the result of the count, vaccine stock bottles of

[1] This is a pure genetic strain of black mice developed by Dr. C. C. Little, director of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. We have used this strain in all our recent experimental work on typhoid vaccine and found it to be highly satisfactory.

8-liter capacity, each containing 5 liters of sterile buffered saline, several 1-liter and 2-liter flasks of sterile buffered saline, sterile 1,000-cc. cylinder graduates, sterile 10-cc. graduated pipettes, and a bottle of tricresol. For each of the 2-liter flasks of concentrated suspension, a suitable number of the 8-liter stock bottles is provided. Each of these has been tagged to show the calculated amount of the various components these stock bottles are to receive. The information on the tag is recorded as follows:

TYPHOID VACCINE

No .... Section ....
Date made ....
Amount of tricresol cc
Amount of suspension cc
Amount of salt solution cc
Total amount of vaccine cc
Standardization: 1,000 million per cubic centimeter.

In making the dilution, the officer who does this work notes first the amount of tricresol required and measures the proper quantity into the 5 liters of sterile saline contained in the 8-liter stock bottle. The bottle is then shaken until thorough solution of the tricresol has been effected. Next the quantity of saline needed beyond the 5 liters is added from the 1-liter and 2-liter flasks provided. Finally, the amount of concentrated suspension shown on the tag is measured into the stock bottle, using for this the sterile graduated cylinder. After thorough agitation the bottle of diluted vaccine is set aside at room temperature for 48 hours. At the end of this time sterility tests are made. These consist of planting two fermentation tubes containing National Institute of Health sterility broth. If, afer 96 hours' incubation no bacterial growth can be detected in any of the fermentation tubes, the vaccine is ready to be pooled.

Pooling.—In order that minor differences which may occur in the specific potency of various small lots of vaccine may be minimized, the contents of twenty to twenty-five 8-liter stock bottles are mixed or pooled. For this part of the work one cubicle is used exclusively. In it are the mixing chamber and a steam generator. The mixing chamber is a heavy monel metal barrel, so set that it can be rotated on its axis, while inside it are baffles which aid in the thorough mixing of its contents. In its resting position it has, at its upper and lower sides, threaded tubulature. The upper is closed by a cap which may be removed to be replaced by the steam-hose connection for sterilization or by a hooded sterile funnel to receive the vaccine which is to be pooled; the lower opening is protected by a bell-shaped funnel under which sterile bottles are placed to receive the pooled vaccine through a large stopcock. For sterilizing the chamber an arrangement of checkvalves, like those on autoclaves, is attached to the lower opening; this insures the escape of all air and condensation water, thus making the process of sterilization of the chamber identical with that of an autoclave. The special generator produces steam rapidly and in ample amount; a heavy, high-pressure hose conveys the steam to the upper opening. The chamber is sterilized for 20 minutes at 15 pounds pressure, just long enough beforehand to give it time to cool before the process of pooling is begun.

With the hooded funnel in place and the cut-off valve below closed, vaccine is carefully poured in at the top, using flame technique. This completed, the barrel is turned back and forth by means of a large crank to mix

thoroughly and insure uniformity of strength of the contained vaccine; then an air filter is attached to the upper opening, and the vaccine is drawn off into sterile 8-liter stock bottles, placed in succession under the funnel below.

These filled stock bottles are stoppered and labelled and their contents tested for sterility. The sterility tests are identical with those made on the vaccine 48 hours subsequent to dilution.

Biological tests—Six animals are used in testing each lot of vaccine. Four mice each receive 0.5 cc. of the vaccine intraperitoneally; a guinea pig receives 0.5 cc. intraperitoneally for each 100 gm. of its weight; a rabbit receives three doses—the first 0.5 cc. subcutaneously, the second, 7 days later, 1 cc. intravenously, and after 7 more days 1 cc. intravenously. Ten days subsequent to the third dose the rabbit is bled and the specific agglutinin titer of its serum is determined. It is generally between 1:5,000 and 1:10,000. The agglutinating suspension consists of living bacilli (500 million per cubic centimeter) suspended in physiological saline. The tubes are incubated at 56°C. for 2 hours and stored in the refrigerator overnight. Complete agglutination only is read. These tests for agglutinins in the blood of the rabbit are intended only as a check on the identity of the bacilli in the vaccine. The mice and the guinea pig serve as checks on the tricresol content and the presence of any directly toxic or infectious substance. These animals are observed for 10 days. All animals dying within this period are carefully examined to determine the cause of the fatality. It must be ascertained whether or not death resulted from an intercurrent infection or from some cause inherent in the vaccine. If death from intercurrent infection should happen to be excluded, the tests are carefully repeated.

The reaction of each lot of vaccine is determined colorimetrically. Phenol red, 0.5 cc. of a 0.02 percent solution, is added to 10 cc. of vaccine. The buffer in the suspending saline solution holds the reaction at about pH 7.2. As a check on the quality of the glass, that is, to ascertain whether or not soluble substances in the glass are affecting the reaction of the vaccine. the pH of retained samples is investigated several months after filling.

Bottling—The vaccine is distributed in 1-cc. ampules and in 5-, 10-, 25-, and 50-cc. vials. These are received at the filling cubicle in the metal boxes in which they were sterilized. In actual use the sides are closed by sterile towels wet with 3-percent cresol solution.

The stock bottle of vaccine is inverted above the sterilized filling cabinet after having been fitted with a rubber stopper which has two glass tubes running through it. One of these, the air-inlet tube, reaches nearly to the bottom of the bottle and is connected outside with a phenol solution air-washing bottle and a sterile cotton air filter. The other, the filling tube, is short, ending just inside the stopper. As the vaccine runs out through this tube, it passes by means of suitable rubber tubing through a sterile gauze filter, then through another rubber tube which enters the filling cabinet. The end of this tube has inserted into it a small section of glass tubing tapering to form the filling nozzle. A pinch-cock clamps the rubber just above this end piece.

When the stock bottle of vaccine is in place, a tray of sterile ampules or vials is passed into the sterilized filling cabinet, then the technician, wearing sterilized clothing and rubber gloves, passes his hand into the cabinet through stockinette sleeves and fills the small containers one after

another by manipulating the pinch-cock. The tray of filled containers is removed and stoppered, another tray immediately taking its place.

Final sterility tests—The vaccine, bottled for issue, is stored at room temperature for 48 hours. Then sterility tests are made upon a random sample taken from a number of bottles. The number of bottles in the sample depends somewhat upon the number filled from the lot of vaccine. The following schedule is used:

Number of bottles filled:	*Number of bottles tested*
100 or less	3
101 to 150	4
151 to 200	5
201 to 250	6
251 to 300	7
301 to 350	8
351 to 400	9
Over 400	10

From each ampule or bottle at least two fermentation tubes are planted; one receives 0.25 cc., the other 1 cc. of the vaccine. Care is exercised in the inoculation procedure to introduce the vaccine into the closed arm as well as into the aerobic chamber of the fermentation tubes.

Prior to inoculation all fermentation tubes are placed in an Arnold sterilizer for 30 minutes at 100° C., cooled to room temperature and planted immediately. Each tube is carefully inspected for air bubbles in the closed arm. If present, the tube is not used. The fermentation tubes are incubated at 37.5°C. for a period of 7 days. They are inspected at the end of 48, 96, and 168 hours. The vaccine may be released for distribution or shipment only if every tube is free of all evidence of bacterial growth.

Retained samples—When a lot of vaccine is bottled for distribution, a number of the ampules and vials from that particular lot are withdrawn and stored for an indefinite period of time. When the contents of one 8-liter stock bottle is transferred into ampules and vials, the number of samples retained consists of five 1-cc. ampules, two 5-cc. vials, one 10-cc. vial, one 25-cc. vial, and one 50-cc. vial. These samples are then available for future studies, such, for example, as a study to determine evidence of any deterioration which might occur.

Immunogenic potency test—There is at present no generally accepted method by which the relative specific activity of typhoid vaccines may be titrated. The need for such a test is self-evident, and work is now in progress at the Army Medical School which has as its object the development of a method for comparative standardization. The results of these studies will be the subject of a supplementary report."[1]

USES

● As a prophylactic, the vaccine (after being shaken well immediately before use) is administered in three doses, the first containing one-half billion bacteria and the second and third each containing one billion organism per cc. of suspension. For women and children under 150 lbs. in weight, the dosage is to be diminshed proportionately. Children, however, tolerate the injections remarkably well. The injections given at

[1] Protective Antibodies in the Blood Serum of Individuals after Immunization with Typhoid Vaccine. By the Laboratory Staff, Army Medical School, Washington, D. C., under the supervision of J. F. Siler, M.D., Am. J. Pub. Health, 27(2):142 (February 1937).

intervals of a week or ten days, are administered subcutaneously, employing the area below the insertion of the deltoid or in the back or abdominal walls. Intradermal injections are advocated by some as being more effective and resulting in less after-effects. Deep and intramuscular injections should be avoided. Smaller doses, but the sum total being the same number of organisms may be given if more time is available, so as to lessen the risk of reactions. Inoculated persons should abstain from severe exercise for twenty-four hours following an injection. It is well to administer a cathartic the day before the inoculation is made. Severe local reaction may occur in a small percentage of cases, especially if the patients are in a poor physical condition. By administering the bacterial vaccine in the late afternoon (3 to 5 P.M.), the reaction develops and appears during the night and is thus apt to be less disturbing. The local reaction usually consists of a very large red and tender area lasting about forty-eight hours. At times the pain and edema may be more marked but abscess formation is practically unknown. Severe constitutional symptoms are less common and usually disappear quickly. The general reaction consisting of headache, malaise and at times giving rise to fever and chills and occasionally to nausea, vomiting or diarrhea is more infrequent and milder in children than in adults.

"Vi" Antigen

Felix and Pitt (*Lancet, 2,* 186 (1934)) detected a new antigenic factor in freshly isolated, actively virulent cultures. This substance was suggested due to the fact that freshly isolated living typhoid bacilli were not agglutinated by an anti "O" serum. A "Vi" antibody was produced which possessed a powerful protective action. The "O" and "H" antigens are not affected by the "Vi" antibody and the latter is incapable of neutralizing the endotoxin produced by the organism, this being the function of the "O" antibody. The "Vi" antigen though a factor found in virulent organisms is not *per se* responsible for the virulence. The latter is due to a combination of both the "Vi" and the "O" antigens. Experimental evidence indicates that antisera prepared from "natural Vi" antigen or "formolized Vi" antigen may be of value as a therapeutic agent, (*Brit. J. Exp. Path., 15,* 346 (1934) ; *16,* 422 (1935)). The fact that "Vi" antigen is sensitive to heat and certain chemicals would appear to challenge the effectiveness of typhoid vaccine as prepared today. Extensive experiments however have proved the value of the latter (see also *J. Hyg., 6,* 559 (1936)).

SENSITIZED TYPHOID VACCINE

● Some workers have advocated and used sensitized typhoid vaccines (so-called typhoid serobacterins) prepared in the same manner as the plain saline suspensions except that the living bacilli (before they are heated) are mixed (sensitized) with their immune serum (antityphoid serum) and after a short period of incubation, the mixture is centrifugalized, the serum discarded, and the bacterial sediment is washed (to remove serum products) and resuspended in saline, heated and the procedure is the same as in the production of the plain bacterial saline suspension. Sensitized heat attenuated typhoid vaccine is said to produce less severe reactions and some even claim that it is more protective than the plain typhoid vaccines.

LYSED VACCINE

● Frawley (*Cal. & West. Med.*, *48*, 415, 1938)) gives Caronia's method of preparing a lysed vaccine which was used successfully in the treatment of typhoid fever in children, a method of treatment which originated in Italy. The product used is prepared by incubating 100 cc. of *E. typhi* culture with 5 cc. of typhoid convalscent blood, until all or nearly all of the bacteria are lysed. The clear liquid portion of the lysed culture is obtained and phenolized.

ORAL ADMINISTRATION

● Besradka (*Local Immunization*, translated by H. Plotz (1927)) has recommended the oral administration of dead typhoid bacilli preceded by doses of dried ox-bile. It is thus possible to do away with hypodermic injections and resulting reactions and he claims that the resulting immunity offers more protection than is afforded by subcutaneous injection, due to the fact that oral administration produces in addition a local immunity of the small intestine so that the impermeability of the intestinal mucosa closes the portal of entry. Three doses (each pill, capsule, or cachet containing some 45,000 millions of dried bacilli killed by heat) are given before breakfast on three consecutive days, and these pills are preceded by requisite doses of pills of dried ox-bile. This method has been used on a wide scale in some European and African countries and in this country it has been employed by Hoffstadt and her associates (*Am. J. Hyg.*, *9*, 1-37, (1929)). Though favorable results have been reported, further observations are needed and a wider application of this method must be exercised before definite conclusions can be forthcoming. Blaizat (*Biol. med. Paris*, *23*, 132 (1933)) presented a review of the extensive literature on this subject.

"Typhoral", an oral typhoid vaccine, and *"Typhoral Mixed"*, an oral typhoid-paratyphoid vaccine, are marketed for immunization by mouth. The former consists of a red pulvule containing 10,000 million heat-killed typhoid bacilli. Three such pulvules constitute an immunization. "Typhoral Mixed" consists of three pulvules, each containing 10,000 heat-killed typhoid bacilli and 5,000 million each of paratyphoid A and B heat-killed bacilli. Children are given the same doses as are adults. The pulvules (or contents) are given with water on an emtpy stomach three successive mornings one hour before eating. A capsule (green colored) containing 1½ grains powdered extract ox-gall is given previously to the first dose of both vaccines, allowing a lapse of 15 minutes between the administration of both capsules. It is recommended that oral vaccination, when practiced, should be carried out annually. The oral method may find more favor with some individuals, who presenting themselves for typhoid-paratyphoid prophylaxis, do not want to be 'stuck'. Oral vaccination appears to give a lessened reaction (and if present usually a local intestinal disturbance) than apparent by the parenteral route.

Workers in this country prefer the subcutaneous immunization procedure and it does not appear that the oral method will supplant it.

NEGATIVE PHASES OF TYPHOID VACCINATION

● Inasmuch as the injection of typhoid vaccine results in the presence of a "negative phase" before immunity is developed, Gerundo (*J. Kansas M. Soc.*, *39* (1938)) advises that *immediate* contacts of typhoid fever cases, who may already be infected, be quarantined without vaccination, while

other individuals in the community be vaccinated. The assumption is that individuals actually in the incubation period of the disease may at that time have their resistance lowered by the inoculation.

PROTECTION AFFORDED

● There is no absolute information upon which opinions can be based as to the duration of the immunity by typhoid inoculations. The degree of immunity is however high yet on the other hand, typhoid immunization is not to be regarded as a COMPLETE protection at any time. The degree of exposure and the conditions of life and body health are factors to be considered. The protection however is powerful and sufficient to prevent a possible contraction of typhoid fever from the ordinary chance infection. The usual custom for the civilian population is to repeat the injections every two years. For soldiers in the field, after the first series of three doses, single or double doses are to be repeated every six months. Individuals coming in contact with typhoid patients and those proceeding to localities where typhoid fever is endemic should be immunized. Persons travel today more so than ever before by automobile, bus and by train to remote places. Tents are pitched and camps are established in inaccessible quarters and individuals imbibe drinking water and they swim in streams, the pollution or purity of which is not known to them. It is certainly advisable that such vacationists and travelers be immunized against typhoid and paratyphoid fevers, allowing ample time for immunization to become effective before proceeding on their sojourns. If after the elapse of a period of time after typhoid immunization, typhoid agglutinins are still present in the blood (diluted 1:100 or higher) the individual will probably not contract typhoid fever from the ordinary case of this disease. It is advisable that people in areas stricken by floods should be immunized against typhoid fever.

The U. S. Army Medical School workers (*Am. J. Publ. Health*, *29*, 95 (1939)) conducting antibody studies on individuals vaccinated against typhoid fever found that considerable immunity is present 10 years after vaccination, but that re-vaccination is desirable two to four years after vaccination. Re-vaccination with a single intracutaneous dose of 0.1 cc. of typhoid vaccine two to ten years after the first vaccination gave an immediate immunological response which was comparable to that obtained with initial vaccination of 3 doses, (0.5, 1 and 1 cc.). The single-dose method of re-vaccination is recommended by them. Substantiating these findings may result in changing the present technique of re-vaccination. For its value and curative properties, see under "Triple Vaccine", the description of which follows.

XV

VACCINUM TYPHO-PARATYPHOSUM
TYPHOID-PARATYPHOID 'A' AND 'B' VACCINE

Bacterial Vaccine Made From the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli

Vac. Typho-paratyphos—Typhoid Combined Vaccine, Typhoid-Paratyphoid Combined Vaccine, Typhoid Mixed Vaccine Prophylactic, Typhoid-Paratyphoid Prophylactic, Mixed Enteric Vaccine

"BACTERIAL Vaccine made from the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli is a suspension in physiological solution of sodium chloride of killed typhoid bacilli (*Eberthella typhi*) and killed paratyphoid "A" bacilli (*Salmonella paratyphi*) and killed paratyphoid "B" bacilli (*Salmonella schottmulleri*).

The Vaccine shall contain in 1 cc., at least 1,000,000,000 typhoid organisms and at least 500,000,000 of each of the paratyphoid organisms.

Description and Physical Properties

● A more or less turbid, whitish fluid; nearly odorless or having a faint odor due to the presence of a preservative. Bacterial Vaccine made from the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli must be sterile and must not contain an excessive amount of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol when either of these is used).

Bacterial Vaccine made from the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli must be prepared in an establishment licensed by the Secretary of the Treasury of the United States.

The outside label must bear the name *Bacterial Vaccine made from the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli*, the number of each of the organisms represented in 1 cc., the lot number of the vaccine, the name, address, and license number of the manufacturer, and the date beyond which the Vaccine may not be expected to retain the potency prescribed by governmental authority.

Storage

● Preserve Bacterial Vaccine made from the Typhoid Bacillus and the Paratyphoid "A" and "B" Bacilli at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass containers in which it was placed by the manufacturer.

Average Dose

● Prophylactic, by hypodermic injection, 0.5 cc. and 1 cc., the latter dose to be repeated once."

Typhoid fever and paratyphoid fever are similar in their clinical manifestations and laboratory aids are required for a correct diagnosis. Though the etiological factors (causative agents) of both are closely related, one does not produce an immunity against the other. Investigations have revealed that about 3 percent of the cases of so-called "mild clinical typhoid" are in reality paratyphoid infections. During the U. S. Army mobilization on the Mexican Border in 1916, after all the soldiers stationed here were inoculated with typhoid vaccine, there developed several small outbreaks of partyphoid fever. It has therefore become the common practice to immunize those in the military service and others with attenuated bacteria which will afford protection not only against typhoid but also against paratyphoid fever.

"Typhoid Vaccine Combined", known also as the "Triple Vaccine" or "T.A.B.", official in the British Pharmacopeia as "Vaccinum Typho-Paratyphosum (Anti-typhoid-paratyphoid Vaccine)" was used during the World War and is used almost exclusively today in civil practice as well as among all military forces. Triple vaccine contains in each cc of suspension, 1,000,000,000 of B. *typhosus* (*Eberthella typhi*), 500,000,000 or 750,000,000 of *B. paratyphosus A* (*Salmonella paratyphi*) and 500,000,000 or 750,000,000 of *B. paratyphosus B* (*Salmonella schottmulleri*). In preparing this vaccine the paratyphoid A and paratyphoid B bacterial cultures and suspensions are made separately and in the same manner as described under typhoid vaccine. *B. paratyphosus* A ("Kessel" strain) isolated from a case among the New York militia troops on the Mexican border in 1916 and *B. paratyphosus B.* ("Rowland" strain), the English army strain isolated from a case in Flanders in 1915 are the strains most frequently employed in preparing this vaccine for marketable purposes. After dilutions of the three suspensions are made separately in the laboratory, they are then mixed together in such proportions as to provide the desired concentration of each in the finished product, as previously mentioned. As a prophylactic, three injections are given.

USES—FOR PROPHYLAXIS

● The first dose is 0.5 cc. and the next two doses are 1 cc. each, injections being given at intervals of 7 to 10 days as described under Typhoid Vaccine. The dosage is to be diminished for children and women according to weight. For its use in oral immunization, see under Typhoid Vaccine.

In administering typhoid or triple vaccine as a prophylactic the preparation is to be injected subcutaneously and care is to be observed that it is not injected into a small vein. A thermal reaction and chills may result by such an accident. Local reactions are frequent. Systemic reactions vary in individual cases and are more apt to occur after the second or third injection than the first one. An interesting study on the fever-producing principle in typhoid vaccine was presented recently (*Proc. Soc. Exptl. Biol. Med.*, *41*, 272 (1939)).

Typhoid and paratyphoid fevers, diseases occurring spontaneously only in human beings have been diminishing in frequency in civilized countries since the beginning of the present century. They are however still formidable causes of death and disability rates. The morbidity and death rates for these diseases vary according to the extent to which sanitary measures in the community have been developed. Immunization against typhoid and paratyphoid fevers is not to be used as a substitute for these sanitary measures, but where the latter are not being practiced at all or to the

degree which they should be or where minor sources of contamination are always possible antityphoid vaccination will afford a large degree of protection.

VALUE

● The value of typhoid and paratyphoid prophylactic therapy has been definitely established. Pfeiffer and Kolle were the first to immunize human beings against typhoid fever in 1896. In 1898, Wright inoculated men in the English Army in India and later in South Africa on a large scale. Within a period of four years, almost 100,000 cases were handled under his supervision. In 1909, Russell began and in 1911 immunization against typhoid was made compulsory in the United States Army. Colonel Russell's report of Dec. 20, 1919, gives an interesting story which well illustrates the efficacy of this prophylactic treatment. The excellent results reported by the universal use of antityphoid immunization during the World War in spite of the most insanitary conditions in many of the battlefields and some of the war fronts more than justifies the extensive use of this practice. Immunization against typhoid reduced the morbidity at least 7 times and the mortality 8.5 times than during peace periods and this does not take into consideration the insanitary conditions and aggravating influences prevailing during that war. Of the four million men in the U. S. Army during the World War, one out of 3,756 developed typhoid fever and one out of 25,641 died. During the Spanish-American War (147,795 men serving), before the universal practice of typhoid prophylaxis, one out of 7, (20,926 cases) had typhoid and one out of 67 died (2,192 deaths and this does not include the number of unrecognized cases). If the same prevalence of cases had existed, there would have been approximately 600,000 men sick with typhoid fever and 60,000 deaths due to the latter during the World War.

THERAPEUTIC OR CURATIVE USE

● Some workers have reported favorable results in the use of typhoid and triple vaccines in the treatment of some chronic and even acute illnesses. Its use intravenously has been advocated to provoke general systemic reactions in the treatment of typhoid fever, disseminated sclerosis, rheumatoid arthritis, severe cases of acne, pneumonia, tertiary syphilis, general paralysis and in certain eye diseases as severe, acute and sub-acute infections of the conjunctiva, ulcers of the cornea and keratitis. Any worthwhile effects appear to be purely non-specific and in which antibody production plays no part. The most notable changes are the affect upon the leucocytes and general cellular stimulation. Great caution must be exercised in the use of typhoid or triple vaccines for curative purposes and until more information is made available, their use as therapeutic agents are not to be generally advocated.

Crouch (*Reprint 1418, U. S. Public Health Reports,* 1930) in a recent study of the effect of typhoid vaccine when given after infection (typhoid fever) has set in arrives at the following tentative conclusion: "That typhoid vaccine is well worthwhile when given after the infection is received, provided the first dose is given before the onset of symptoms and provided further that three doses are given." Administered soon after the onset of symptoms, the vaccine is of little or no benefit in the treatment of typhoid fever. Crouch employed the marketed "triple vaccine" and the therapeutic doses were one-half the usual prophylactic dose, administered at three or four day intervals.

Kapuscinski (*Soc. franc. d'Opthalmologiew,* 51st Congress, 5/16-19, (1938)) on the basis of results in 50 cases treated with typhoid vaccine as well as hematological observations recommends typhoid vaccine as the best pyrotherapeutic agent in ophthalmology. He reports on many opthalmological affections which responded effectively to pyrotherapy with typhoid vaccine.

Of interest is a recent report in which subcutaneous injections of a typhoid bacillus filtrate led to the disappearance of multiple warts in 196 of 200 cases. The warts had been of three to fifteen years duration and had proved refractory to all other procedures. One or two courses of 6 injections were given. The warts began to show changes as early as after the third injection and disappeared in all successful cases within 2 weeks following the last injection. There were no recurrences.

XVI

OTHER BACTERIAL VACCINES

WHOOPING COUGH VACCINE (VACCINUM PERTUSSIS)

SAUER (*J. A. M. A.*, Nov. 4, 1933), has prepared a pertussis vaccine which is a modification of that devised by Madsen and Associates (*Centenary Meeting*, Brit. Med. Assoc., 1932) at the Danish Statens Serum Institute. This is made from recently isolated hemolytic strains of *H. pertussis* (Bordet-Gengou bacillus) and grown on Bordet medium containing 20% fresh defibrinated human blood. The growth is subjected to agglutination and absorption tests to prove that the strain belongs to Leslie and Gardner's Phase I (*J. Hygiene, XXXI,* 423, (1930)). The finished preparation contains 10 billions bacilli per cc. of suspension which are killed by the addition of 0.5% phenol and exposure in the refrigerator for one week, during which time the suspension is agitated daily. As a prophylactic it is used in three doses (0.5 cc., 1 cc. and 1.5 cc.), subcutaneous or intramuscular injections being given at intervals of 5 to 7 days. Sauer further reports: "Active immunity is completed in four months and lasts for years. Infants withstand the injection remarkably well. The best age for immunization is the second half year of life." In the *British Medical Journal* (Dec. 16, 1934, 1109), an editorial expressed a doubt concerning the efficiency of other types of vaccines as compared to the Sauer and Madsen types in the prophylaxis of whooping cough. Sauer and other workers recommend vaccine therapy for the latter affection not only for protection but for treatment as well especially in the catarrhal stages of the disease (*Med. Klin.*, 1934), (*J. Pediat.*, *5* Aug., 1934, 244-273).

Sauer Pertussis Vaccine is to be kept refrigerated at 2°C. to 12°C. at all times. Vaccine that has been frozen or allowed to remain outside of the refrigerator for long periods of time should not be used. To avoid the injection of a chilled vaccine, the latter is to be allowed to stand for a few minutes at room temperature before administration. The unused portion of the vaccine is to be returned to the refrigerator promptly.

Pertussis prophylactic immunization with adequate dosage of a potent vaccine appears to be of sufficient value to warrant its wide use (*Am. J. Dis. Children, 54,* 979 (1937)). Dosage schemes other than the above have been used. Kramer (*J. Pediatr., 12,* 160 (1938)) employed in his vaccination course subcutaneous injections of 1 cc. into each arm, followed by 1.5 cc. in each arm 7 days later and a final 1.5 cc. in each arm 7 days

after the second dose. This total dosage of 8 cc. is recommended by other workers using the deltoid, triceps, and biceps regions as repeated injections are to be avoided in the same area. To reduce to a minimum the likelihood of reactions, it has been suggested that the total of 8 cc. should be administered in single rather than double injections and doubling the number of weekly intervals over which the injections are given. In highly susceptible individuals where severe reactions follow an injection, the volume of the dose following such reaction is not to be increased.

Smaller doses, and more injections, possibly at more frequent intervals, are to be given. Immunization should be completed as soon after the 6th month of the infant's life as possible.

Blatt and associates (*J. Pediatr.*, *12*, 619 (1938)) suggest a formalized vaccine and Leonard (*J. M. Soc. N. J.*, *34*, 451 (1937)) recommends a slightly modified Sauer Vaccine for immunization. Harrison and Associates (*Publ. Health Reports*, *53*, 793 (1938)) report on the use of a single dose of precipitated pertussis vaccine and suggest the further clinical trial on a larger scale to prove the value of this agent as a prophylactic. Many reports reveal that pertussis vaccine is of value in the early treatment of the disease as it lessens the symptoms and shortens the course of the infection. Four or five injections (0.25 cc., 0.5 cc., 1.0 cc., 1.5 cc., and 2.0 cc.) are given at intervals of 3 to 5 days, depending upon the appearance of a reaction. This dosage is generally reduced to one-half for children under one year of age. Intranasal administration of an antigen prepared from cultures of *H. pertussis*, both in the treatment and in the prophylaxis of whooping cough has been reported and the results warrant further trial of such products (*J. Mich. M. Soc.*, *36*, 849 (1937); *Penna. M. J.*, *41*, 485 (1938)). Toomey (*Ohio State M. J.*, *33*, 161 (1937); *J. Pediatr.*, *10*, 472 (1937)) discusses all phases of the whooping cough problem, its prophylaxis and treatment, and considers the value of many of the antigens as well as of convalescent serum.

Most workers are in agreement that substantial protection is afforded by a properly prepared and properly administered whooping cough vaccine (*J. A. M. A.*, *112*, 1145 (1939); *Am. J. Hyg.*, *29*, Sec. B. 133 (1939)). On the basis of the present evidence, a vaccine prepared from virulent *H. pertussis* of complete antigenic structure appears to be of value if properly administered as a prophylactic agent.

Lechner (*Brit. Med. J.*, *1*, 1154 (1939)) is in favor of large doses of whooping cough vaccine as a therapeutic agent. He recommends 3 injections of 5, 7½, and 10 billion organisms at 2 to 3 days intervals. One-quarter of these doses are used for children under 2 years of age.

Pertussis Topagen—*Pertussis Soluble Antigen* is a sterile buffered saline solution of soluble antigenic substances prepared from freshly isolated Phase I cultures of *H. pertussis* to which a preservative is added (ortho chloro-mercuri phenol 1:20,000). Each cc. represents the antigenic fraction of 20 billion organisms. It is standardized by complement-fixation testing against specific immune serum. It is used mainly as a therapeutic agent and application is by intranasal instillation in 0.25 cc. doses daily or every other day for a period of at least two weeks. The preparation must be applied to the area of the middle and superior turbinates. Favorable reports have been presented in the use of this preparation as the sole therapeutic agent in approximately 100 cases (*J. Pediat.* *9*, 42 (1936); *10*, 641 (1937)); (*J. Mich. M. Soc.*, *36*, 849 (1937); *Penna. M. J.*, *41*, 485 (1938)).



Collecting blood aseptically from a rabbit in process of biological manufacture.

Diluting concentrated suspensions of bacterial vaccine to standardized bacterial count.





Final step in the preparation of Pertussis Vaccine (Sauer). Standardization to 10 billion organisms per cc.

HAFFKINE'S VACCINE; PLAGUE BACILLUS VACCINE; ANTI-PLAGUE VACCINE

VACCINUM BUBONICUM; VACCINUM PESTIS

This is employed especially in India as a prophylactic against plague. It consists of strains of *P. pestis* originally isolated by blood culture from a human case of septicemic plague grown in neutral broth in shallow containers to which a few drops of sterile olive oil or butter fat is added. They are incubated for 6 weeks at room temperature and shaken once daily. At the end of this period, the cultures are agitated well and heated at 55° to 65°C. for from 15 minutes to several hours (1 to 3 hrs.) This material to which 0.5% phenol is added and consisting of bacterial products, attenuated bacteria and broth protein has been and is being used more than any of the other attenuated suspensions of plague bacilli and appears to possess protective value (*J. A. M. A.*, 1921, *76:*243), (Haffkine Inst. Report for 1927), though the immunity is of short duration, not much over six months (*Bombay Bact. Lab. Rep.* 1913-1916). Recent researches by Schutze (*Brit. J. Exp. Path.*, 1932, *XIII*, 284, 289, 293) reveal that a virulent strain of *P. pestis* is necessary for the production of a good vaccine. One or two injections in doses of from 3 to 4 cc. for adults are given subcutaneously (at intervals of ten days).

Taylor (*Indian J. Med. Res.*, Sup. Ser. No. 27, 1933) advises grouping highly virulent strains of *H. pestis* in a neutralized standard broth medium (acid-digest of goat flesh) at 27°C. for four weeks. The growth is washed in saline solution and sterilized at 55°C. for 15 minutes. Standardization is by a mouse protective test (*Bull. Off. Int. Hyg. Pub.*, *27*, 1534 (1935)). The adult dose given in one subcutaneous injection is 4 cc. In the paper quoted, Taylor presents sufficient evidence which indicates that both the attack rate and the death rate have been markedly reduced by the use of plague vaccine as a prophylactic.

CHOLERA VACCINE

VACCINUM CHOLERAE

This vaccine (*Trans. 7th Cong. Far East. Assn. Trop. Med.*, 1937. *I*, 523) is prepared from attenuated or (heat or chemically) killed *Vibrio comma* (*Sp. Asiatic Cholera*). Extracts of cholera spirilla have also been used. Castellani (*Manual of Tropical Medicine*, 1919) has prepared T. A. B. C. or tetravaccine consisting of mixed agar culture suspensions of typhoid, paratyphoid A., paratyphoid B. bacilli and cholera spirilla. Such a preparation was used in the Serbian Army during the World War. Prophylactic vaccination is said to afford considerably protection and to be of distinct value. Immunization must be repeated at yearly intervals and certainly every two years (Violle, H., *Le Cholera*, Paris, 1919).

Haffkine used living cultures and later killed organisms in the preparation of his vaccine (*Brit. Med. J.*, *2*, 1541 (1895) and *"Protective Inoculation against Cholera"*, Calcutta (1913)). The present vaccines are prepared from saline suspensions of 24 hour old cultures which are sterilized at 53°C. for one hour (*J. A. M. A.*, *76*, 243 (1921)). The final preparation contains 8 billion organisms per cc. It is administered subcutaneously in two doses of 0.5 cc. and 0.1 cc. Immunization against cholera has been and is practiced extensively in Europe and Asia. Though it is difficult to appraise its true value, it appears that its use as a prophylactic agent is of value.

XVII

BACTERIAL CULTURE FILTRATES, IMMUNIZING TOXINS AND TOXOIDS

FILTRATES containing only the unaltered products of bacterial growth have been and are being employed in the treatment of affections caused by various organisms. Favorable results have been reported with the employment of these filtrates in the treatment of furunculosis, carbunculosis, deep pustular acne, infections caused by suppurative bacteria following cuts, wounds, surgical operations, etc.

PREPARATION OF FILTRATE VACCINES

● Autogenous as well as stock preparations are employed. In the preparation of such an autogenous filtrate, it is customary to inoculate the material from a lesion into a meat-infusion, hormone or brain broth, incubate this at 37°C. for from 4 to 7 days; and filter through a Berkefeld or Mandler filter. Some workers mix this autogenous filtrate with an equal quantity of a stock filtrate prepared in the same way and from the same species of organisms, but using polyvalent stock mixtures. Sterility tests are conducted (by culturing 0.5 cc. in broth for 24 hours), and then 0.5% phenol or other suitable preservative is added. The finished preparation must be kept in a refrigerator. Injections beginning with 0.05 to 0.1 cc. and increasing thereafter by 0.1 cc. are given intramuscularly, subcutaneously, or intradermally at intervals of 4 to 5 days. Autogenous bacterial filtrates are administered by some workers with autogenous bacterial vaccines, either in the same injection or usually alternating with the filtrate and vaccine separately.

Intracutaneous inoculation of filtrates and even of bacterial vaccines is practiced today. Though producing greater reactions this method of inoculation seems to be more productive of and more rapid in antibody response, as recently revealed by Besredka (*Immunity in Infectious Diseases*, Paris) and others. Among the many stock bacterial filtrates marketed and employed intradermally for active immunization, a gonococcus filtrate (Corbus-Ferry) (*J. Immunol.*, 1931: *XXI*, 233) has received the attention of many workers, (*J.A.M.A.*, 1932, *98*:532; 1935, *104*:181). Favorable therapeutic experiences in the treatment of all gonorrheal infections have been reported with this preparation.

TOXINS AND TOXOIDS

UNDER suitable conditions the filtrate of many organisms has been found to possess true soluble toxins which are antigenic. It is believed that these exotoxins are largely responsible for the pathological changes which occur in certain affections. The filtrates containing these toxins have been employed also as diagnostic aids along lines similar to the use of diphtheria toxin in the Schick test. The toxins themselves or the latter changed to toxoids prepared in a manner similar to those of diphtheria and tetanus toxoids have been and are being employed for active immunization. The following are available:

TOXINUM DIPHTHERICUM DIAGNOSTICUM

DIPHTHERIA TOXIN FOR THE SHICK TEST.

Toxin Diphtheric Diagnost.—Shick Test Toxin.

"DIPHTHERIA Toxin for the Schick Test is a solution of the toxic products of growth of the diphtheria bacillus (*Corynebacterium diphtheriæ*).

Description and physical properties—A transparent liquid which should be adjusted to suitable concentration, at the time of administration, by dilution with physiological solution of sodium chloride or other diluent so that one-fiftieth of the minimum lethal dose of Diphtheria Toxin for the Schick Test is contained in 0.1 cc. The minimum lethal dose of Diphtheria Toxin for the Schick Test is defined as the smallest amount of toxin that will cause the death within ninety-six hours of a guinea pig weighing between 250 and 280 Gm. after subcutaneous administration. The product must be sterile. Diphtheria Toxin for the Schick Test must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Diphtheria Toxin for the Schick Test*, the manufacturer's lot number of the Toxin, the name, address, and the license number of the manufacturer, and a statement of the date beyond which the Toxin may not be expected to retain the potency prescribed by governmental authority.

Storage—Preserve Diphtheria Toxin for the Schick Test at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average dose—For determining susceptibility (Schick Test), intracutaneous, 0.1 cc. of the dilution, representing one-fiftieth of the minimum lethal dose."

● In 1883, Klebs described the presence of bacilli in the pseudo-membranes from the throats of those having the characteristic symptoms of diphtheria. One year later, Loeffler obtained pure cultures of these organisms. They have therefore been known also as Klebs-Loeffler bacilli. In more recent classifications, the *Bacillus diphtheriae* has been placed in a genus, *Corynebacterium*, an indication of the possible relationship of this organism to the actinomycetes rather than to the simple rods.

In 1888, Roux and Yersin proved that the diphtheria bacillus produces a soluble toxic substance. They grew the organisms in broth cultures, removed the former from the culture medium by filtration through a Cham-

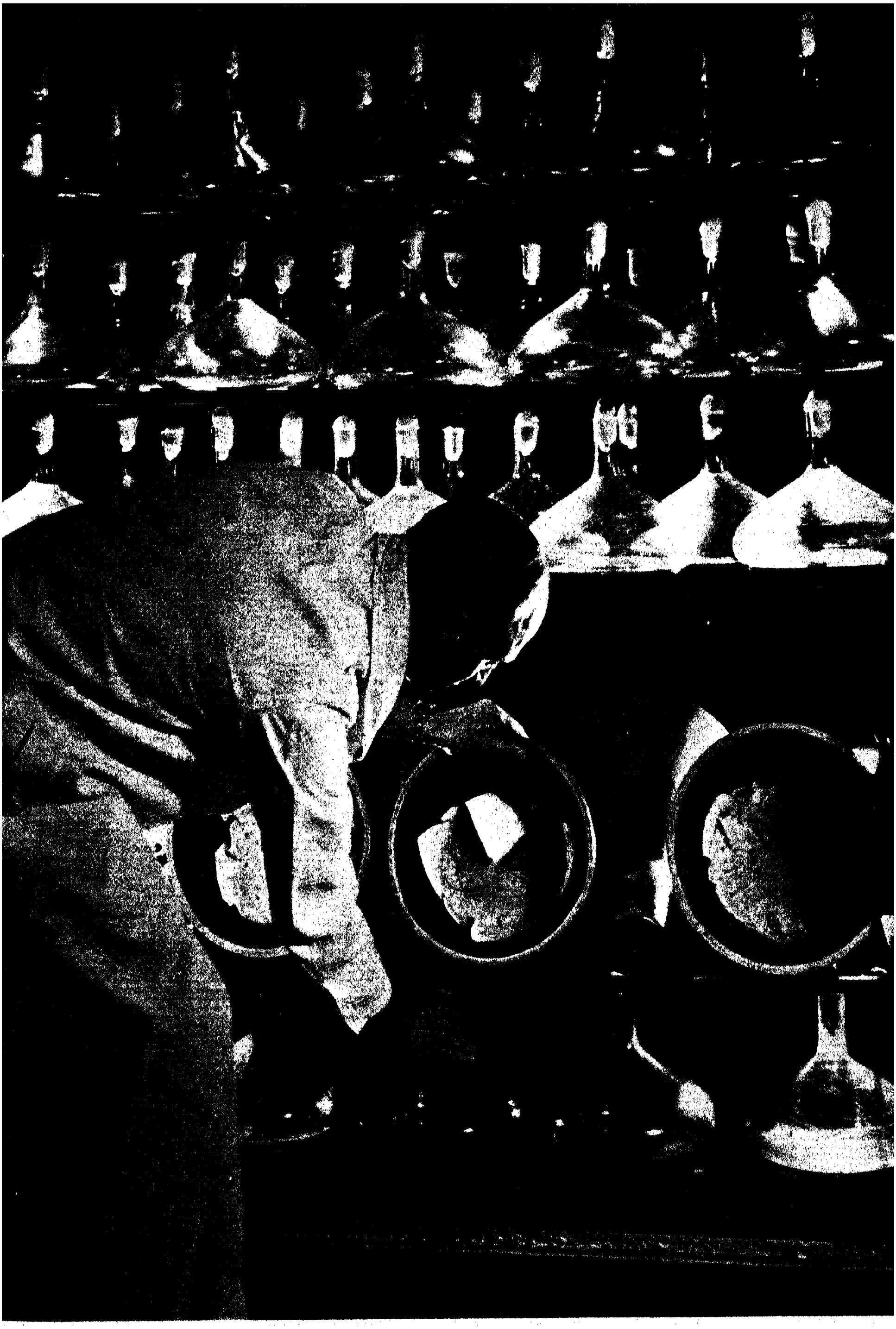
berland filter and found that the filtrate (free of bacilli) was toxic for guinea-pigs. It is this toxin (free of the bacilli, and therefore known as an extracellular, exo or ectotoxin) which in clinical diphtheria ordinarily produces the systemic poisoning and does most of the damage; and it is the toxin which must be combatted when an active diphtheritic infection prevails. Considerable information is available concerning the action of diphtheria toxin upon body tissues, its selective action upon certain nerve centers, (especially apparent upon the vagus, the phrenic and certain cranial nerves), and finally its action upon both the cortical and medullary cells of the suprarenals and to the late degeneration of the heart muscle which is the cause of postdiphtheritic death. The production of a potent toxin is of great commercial import today for this becomes the starting point for the production of all of the other preparations employed either in determining susceptibility to diphtheria or in the prevention and treatment of this infection.

PREPARATION

● The artificial production *in vitro* and the manufacture of toxin for commercial purposes have been found to depend upon a great many different but definite factors and favorable conditions. The requisite conditions for good development of toxin are: A pure culture of *Corynebacterium diphtheriae* but a strain known to yield a potent toxin (as Park and Williams No. 8); a suitable culture medium (as a 2% peptone nutrient bouillon made from fresh veal, using suitable meat (avoid horse meat) and peptone and possessing an alkalinity which should be about pH 7.3 to 7.4); the manner of inoculation of the culture medium (grown in flat-bottom bottles or containers, yielding a shallow, thin layer of medium, with a large surface, thus producing better aeration which will result in a more rapid formation of the maximum amount of toxin); and incubation is to be carried out at the most favorable temperature, between 32° to 35°C. Under such favorable conditions, the greatest accumulation of toxin is produced by the bacilli between the fourth and eighth and usually on or about the seventh day. At this time phenol solution is added to give an approximately 0.5% concentration and the liquid cultures are filtered, as the number of viable organisms rapidly diminish and the conditions for those remaining alive are not suitable for the rapid production of toxin. A description of the production of diphtheria toxin together with a consideration of changes and interesting data concerning Park and Williams' No. 8 organism is given in *Ann. Inst. Past., 61,* 791 (1938). The toxin already produced is not very stable at 35°C. so that the deterioration taking place in the toxin produced is greater than the amount of new toxin likely to form. Filtration of toxin is carried on in a draft-free room and in which the glass sides are painted to exclude the light rays most active in hastening the deterioration of diphetheria toxin. After concentration, the usual sterility and safety, potency, and clinical tests are conducted. The exact chemical composition of such commercially prepared toxin is not definitely known. On the other hand much is known of its stability, behavior toward chemicals and physical agents and its reactions after it is injected into animals. It is a colloid and probably a toxic radical attached to protein (colloid).

ACTION

Dernby (Dernby, K. G.: *J. Gen. Physiol.,* **1926-1927,** *VIII,* 311), advanced the view that the greater part of the diphtheria toxin is neither secreted



Inside of sterilizer showing Fernbach Flasks and filters used in the production of diphtheria toxin.



Close-up of incubator showing Fernbach Flasks inoculated with a culture of the diphtheria bacillus.

from the living cells nor extracted from the dead bacilli, but that the bacilli liberate proteolytic enzymes of a specific character which attack the albumoses and peptones of the broth and especially of the bacilli. The organisms die and autolyze. Some of the primary split products are the toxic-substances but if the proteolysis proceeds too far, these also will be disintegrated.

Diphtheria toxin is not stable, being destroyed or modified rapidly by heating to 60°C. for one-half hour; even heating at 56°C. for one-half hour destroys 95 per cent of it. When concentrated and dried, it may withstand 70°C. Light and oxidation destroy it rapidly. Chemically it is seemingly protein in character, though its protein nature has been questioned. It is non-crystallizable and does not dialyze except through very thin membranes. It is precipitated by ammonium sulphate, alcohol and nucleic acid, so that it can be concentrated and freed from many extraneous materials.

In animals it is found to be a powerful tissue poison. It possesses an affinity for the nervous system of man, the lesion being typically a peripheral neuritis. The paralytic involvement is usually later in the course of the disease. Diphtheria toxin is rapidly fixed by the tissues so that once fixed, it is almost impossible to neutralize it with antitoxin, even when large doses are administered. Injected into laboratory animals there is a delay before changes are apparent. There is a definite period of incubation before diphtheria toxin will kill laboratory animals even when large doses are given, (*J. Path. and Bact.*, *28*, 251 (1925)). Guinea pigs are most suitable for the observations of the action of the toxin, the most characteristic finding being observed in the adrenal glands.

STANDARDIZATION

● Methods have been developed of measuring the degree of toxicity or potency of toxin in terms of the fundamental unit of toxicity. This is the *M. L. D.* or minimum or minimal lethal dose (or M. F. D., minimum fatal dose), which is the smallest amount of diphtheria toxin that when injected subcutaneously will kill a guinea-pig weighing 250 grams at the end of four days (ninety-six hours), with the production of lesions typical of uncomplicated diphtheria poisoning. Other units of measurement are derived from this and apply to results obtained with toxoids or with antitoxin, or with a mixture of toxins and antitoxin (which see).

Diphtheria Test Toxin is so standardized that the amount of toxin injected intradermally (intracutaneously) in one Schick Test dose represents 1/50 M. L. D. The B. P. requires that 0.2 cc. of the preparation shall contain the Test Dose. This dose is measured by noting local reactions as in positive Schick tests obtained in guinea pigs when injected intradermally with 1/25th but not with 1/50 of one Test Dose. Furthermore mixtures of toxin and antitoxin when injected are required to yield the following findings. One Test Dose of Diphtheria Test Toxin mixed with 1/750th or more of a unit of diphtheria antitoxin should produce no local reaction when injected intradermally in guinea pigs while when mixed with 1/1250th or less of a unit of diphtheria antitoxin, a positive Schick reaction should be obtained in guinea pigs. For further details concerning techniques for determining the suitability of toxin for Schick testing, see the Report of the Permanent Commission on Biological Standardization, League of Nations Health Organization (London) 1931 and *J. Immunol.*, *33*, 191 (1937).

Some commercial houses supply Diphtheria Toxin for the Schick Test in a package containing two vials, one ampul containing concentrated diph-

theria toxin glycerinized to prevent evaporation and in the second ampul there is present sterile normal saline solution or other satisfactory diluent. Just before use, a dilution of the proper strength is made. There is however available at present a Schick Test Solution which will retain its potency in diluted form for a number of months. This has been accomplished by using an improved diluent which stabilizes the hydrogen-ion concentration and renders the mixture isotonic with blood. One commonly used contains 0.1% peptone in a borate-boric acid buffer (*J. Immunol., XX,* 71 (1931); *XXII,* 93 (1932)). In these available marketed packages, no manipulation or dilution of the material is necessary and the solution ready for use contains in each 0.1 cc. 1/50 M. L. D. of diphtheria toxin.

Taylor (*Can. Publ. Health J., 30,* 42 (1939)) presents a new Schick toxin prepared by diluting fresh toxin with a 2.7% glycerin solution containing gelatin. She claims that this preparation is more stable and yields more readily interpretable reactions. Sterility tests are made on marketable Diphtheria Toxin Solutions for the Schick Test in accordance with the practice for other biological products. When exposed to 37°C. for 24 hours, marketable diluted diphtheria toxin shall not reveal an appreciable loss in toxicity. It however should be kept at low temperatures at all times and should never be exposed to strong light. The expiration date stamped on each package is six months from the date of manufacture or date of issue for undiluted toxin; one year after date of manufacture or date of issue for properly diluted toxin; the expiration date on the label for both products to bear the provision "If kept between 0° and 8°C. (32° and 46.4°F.)."

USES

● Diphtheria Test Toxin is employed in the Schick Test so as to determine whether one is immune to diphtheria. Injections are given intracutaneously (intradermally), not subcutaneously (*Munch. med. Wchschr., 55, 504* (1908)).

The *Schick test* (*J. Ind. State Med. Assoc.,* 1930, *XXIII,* 320) is used to find out whether individuals have in their blood sufficient diphtheria antitoxin to render them immune to the disease. It is thus possible to determine whether it is desirable to immunize those who are not immune with either antitoxin or toxoid. The technique consists in injecting intradermally (between the layers of the skin) 0.1 cc. of the diphtheria test toxin (containing 1/50 M.L.D.). A positive reaction will be apparent in twenty-four to forty-eight hours, and reach its height in from forty-eight to seventy-two hours, when readings are generally made. This is a circumscribed area of redness about the site of the injection, from 1 to 2 cm. in diameter, which remains from one to two weeks, followed by slight scaling and the remaining of a brownish pigmented area. A control test should be always conducted in persons over five years of age (*Am. J. Pub. Health,* 1934, *XXIV,* 835). The control fluid is injected the same way but in the opposite arm. If the same syringe is used the control fluid is injected first. The control fluid (administered simultaneously and usually injected in the other arm) will detect and properly interpret false or non-specific reactions or combined false and true positive skin reactions. For the purpose of checking reactions, the best reagent to employ is an identical quantity of the same dilution of the toxin as employed in the test proper but this should be heated to 75° C. for at least 10 minutes. Heating destroys the true toxin but does not affect other proteins which may cause false reactions. A "Schick Control" preparations is official in the British Pharmacopeia: "Toxinum Diphthericum Calefactum."

Earle (*J.A.M.A.*, 1934, *103:* 336) has suggested deeper injections instead of the intracutaneous injection as providing a truer index of the immunity status. Simplified methods of performing the Schick test have also been advocated (*Schweiz. med. Wochnschr.*, 1934). Individuals who have received a series of three injections of toxoid or toxin-antitoxin mixture should be tested about two to four months later to see whether an immunity has developed.

In practice, four types of reaction are observed in Schick tests: (1) *Negative,* in which signs of reactions do not appear or are very slight and identical in case of the test and control areas; (2) *Positive,* in which the test area reveals a definite local erythema (1 to 2 cm. in diameter) within 24 to 48 hours which is well marked by the 4th or 5th day. This lasts for 7 to 15 days, then gradually fading, presenting a persistent brownish appearance with or without slight desquamation. The control area shows no or little local inflammation: (3) *Pseudo-reactions,* which are in reality negative. At both sites of injection, definite and even marked urticarial reactions of the allergic type appear within 6 to 24 hours. They are less sharply circumscribed, less intense than the positive reaction, subside very rapidly and are not apparent by the fourth day. The reaction is of a non-specific nature; (4) *A combination of (2) and (3),* so that both areas reveal little difference in the extent of local erythema at the end of 24 hours. Gradually however, the difference becomes more apparent, being striking by the fourth day, when a well-defined positive result is apparent on the test area as compared with the complete disappearance of or the presence of only a slight inflammatory area on the control arm.

Reactions (2) and (4) are positive and indicate susceptibility to diphtheria. Reactions (1) and (3) are negative and indicate the presence of at least 0.01 to 0.25 unit of antitoxin per cc. of blood. This is an amount of antitoxin sufficient to protect from all ordinary risk of infection.

Pieere (*Compt. rend. soc. biol. 125,* 1093 (1937)) discusses delayed Schick reactions as noted in 5 to 6 days after inoculation in 2.6% of approximately 6,000 cases. Kutscher (*South. Med. & Surg., 100,* 237 (1938)) and other workers have advocated repeated Schick tests regardless of the agent used in the immunization procedure. He advises repetition of the test every 2 or 3 years.

Diphtheria Scratch Test

● Grozin, (*Am. J. Disease Children 57,* 564 (1939)) suggests a diphtheria scratch test made with toxin of 1,200 M.L.D.'s and at least 30 flocculating units per cc. The test is performed and read in the same manner as the von Pirquet test for tuberculosis. It is said to be specific for diphtheria and compares favorably with the Schick test, over which he claims it has the advantages of being less time consuming and slightly more sensitive.

TOXINUM DIPHTHERICUM CALEFACTUM B.P.

(Toxin. Diphtheric. Calefact.) Schick Control

Synonyms:—Diphtheria Toxin (Heated) for Schick Test Control; Schick Test Control.

This product heated to a temperature of not less than 70°C. for 5 minutes is made from the same batch of the unheated Schick Test Toxin (Toxinum Diphthericum Diagnosticum B.P.) with which it is mar-

keted or issued for use as a diagnostic agent when employed for the detection of susceptibility to diphtheria. Its preparation and methods of testing for sterility are the same as given for Schick Test Toxin. Heating at 70° C. or higher temperatures for 5 minutes inactivates the toxicity of diphtheria toxin. This Schick Test Control or Heated Diphtheria Toxin for Schick Test Control is injected intradermally simultaneously (but into the other arm) with the unheated Schick Test Toxin. It is thus possible to obtain a more clear cut observation of the effect of the true diphtheria toxin as compared with possible pseudo reactions due to bacterial and broth proteins and other non-specific substances. The absence of a reaction to the toxin (Schick negative) indicates definitely an immunity to diphtheria if the test-toxin is of *suitable* strength and if the injection has been made *intradermally.* If there is any doubt whether a reaction is due to the toxin or to the proteins, it is advisable to consider the reaction as positive. The use of a control will aid greatly in determining the character of the reaction in the older age groups.

Diphtheria toxoid for active immunization should be given to children when the reaction is positive, or when for safety the reaction is considered positive. In children under 2 years the Schick test is not usually performed since during the first 9 months infants may be passively immune from antitoxin derived from their mothers. However, during the second year of life, they usually are not immune.

XVIII

TOXINUM DIPHTHERICUM DETOXICATUM

DIPHTHERIA TOXOID

Toxin, Diphtherici. Detox.—

DIPHTHERIA Toxoid is a sterile aqueous solution of the products of growth of the diphtheria bacillus (*Corynebacterium diphtheriæ*) so modified by special treatment as to have lost the ability to cause toxic effects in guinea pigs but retaining the property of inducing active immunity. The toxicity of the Diphtheria Toxoid shall be so low that five times the dose for the adult human does not cause either local or general symptoms of diphtheria poisoning in a guinea pig within thirty days after its injection into the animal. The antigenic value shall be such that the initial dose for the human shall protect at least 80 per cent of guinea pigs, six weeks after injection, against five minimum lethal doses each of diphtheria test toxin.

Description and physical properties —A clear, brownish-yellow or decidedly turbid liquid having a faint, broth-like odor or an odor due to the presence of a preservative. Unconcentrated specimens must be clear.

Diphtheria Toxoid must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Diphtheria Toxoid,* the manufacturer's lot number of the Toxoid, the name, address, and license number of the manufacturer, and a statement of the date beyond which the Toxoid may not be expected to retain the potency prescribed by Governmental authority.

A concentrated Diphtheria Toxoid may occur in the following form known as:

DIPHTHERIA TOXOID, ALUM PRECIPITATED

Refined Diphtheria Toxoid

This is a turbid white suspension prepared by adding a sterile aqueous solution of alum to Diphtheria Toxoid, washing the resultant precipitate with physiological solution of sodium chloride, and resuspending it in physiological solution of sodium chloride to which a suitable preservative may be added.

Alum Precipitated Diphtheria Toxoid shall bear the additional designation *Alum Precipitated* on the labels of the finished package.

It shall meet the requirements as given above under Diphtheria Toxoid with the exception of the *description and physical properties.* It shall also meet the following requirements:

The human dose of Diphtheria Toxoid, Alum Precipitated when admin-

istered subcutaneously to guinea pigs weighing 500 Gm. shall produce at least two units of antitoxin per cc. of blood serum at the end of four weeks. At least four guinea pigs shall be used for this test, but the blood serum from all guinea pigs may be pooled for the test for antitoxic content.

The finished product shall contain not more than 20 mg. of alum per human dose, the calculation being based on the total amount of alum added for precipitation.

Storage—Preserve Diphtheria Toxoid at a temperature between 2° and 10° C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average dose—By hypodermic injection, prophylactic, 1 cc.

Diphtheria Toxin (as well as toxin secreted by other bacteria) upon long standing will lose its toxicity but will be found to retain its initial power for neutralizing its specific antitoxin. Treatment with chemicals or heat will also bring about this modification. There appears to be no relationship between the toxicity of a toxin and its ability to bind, combine or neutralize its specific antitoxin or its ability to stimulate the production of antitoxic substances or antitoxin, (that is retaining its original immunizing power). Ehrlich explains this by stating that the toxin molecule consists of two portions or groups: One which is known as the toxophore group, which is labile, easily destroyed by chemicals, even by exposure to light and air and which is the carrier of the toxic qualities. The other portion known as the haptophore group is characterized by its stability and its ability to cause the neutralization of the antitoxin, or under proper conditions to act as an antigen and incite the body cells to produce specific antitoxin. The haptophore group does not depend upon the toxophore group to exert its effect. With this available knowledge different methods were instituted to modify the toxic powers of diphtheria toxin (and other toxins), to prepare what is known as Diphtheria Toxoid (or other Toxoids) (or Anatoxin, as known in Europe). The toxoids retain their specific antigenic properties, are capable of producing specific antibodies and will combine with them *in vitro*.

Diphtheria Toxoid or Anatoxin on the market today is a detoxified (non-toxic) diphtheria toxin. The conversion of toxin to toxoid is effected by treatment with formaldehyde (or other chemicals) and incubation or exposure during varying periods of time at different temperatures. For production of toxin, see under *Toxinum Diphthericum Diagnosticum.* Horse meat should not be used in making the broth from which the original toxin is made. To a filtered toxin free of bacilli possessing an L_+ dose of not more than 0.2 cc. and an M. L. D. of not more than 0.0025 cc. and containing no preservative, 0.2 to 0.4% formalin is added. This mixture is incubated at 37° to 40° C. for three weeks or longer, until it becomes atoxic. When no toxic action follows the injection of 5 cc. (five human doses) subcutaneously into guinea-pigs weighing approximately 300 Gm. and which are held under observation for several weeks (at least 30 days), the toxoid preparation is considered atoxic. Toxicity, sterility, safety and potency tests are made on all marketable products.

Toxoids are free of any serum protein which Toxin-antitoxin Mixture contains because of the presence of the antitoxin. Not containing horse serum, toxoids obviously do not sensitize individuals against the latter.

Ramon (*Ann. Inst. Past. 61,* 784 (1938)) suggests a newly prepared medium for the production of toxin, resulting in an increase of the antigenic value of the finished toxoid. Toxoids are stable in their immunizing powers,

do not revert to the original toxin, and are not affected by freezing. It must be noted however that protein from the culture medium (from which the toxin entering the Toxoid originates) is found in toxoids and slight protein reactions especially in older children (over 10 years of age) and adults are possible in those sensitive to such proteins. Such reactions consist locally of induration, with an area of redness extending several inches around the site of injection, together with general reactions as malaise, headache and fever. The symptoms usually disappear completely and rapidly in two to three days. Wet dressings of boric acid are frequently helpful in reducing soreness and swelling. If found convenient, suspicious reactors may be tested to determine if marked reactions will be produced. Injections of 0.1 cc. of diluted Diphtheria Toxoid (1:20) intradermally will reveal if a local area of redness will develop within three days. Diluted toxoid for the reaction test is available on the market in a separate package. If the reddened area produced in an injection is less than one-half inch in diameter, Toxoid injections may be given. If greater reactions prevail, T-A mixture may be employed instead of Toxoid. In young children (under 6 years of age) protein reactions attending the use of toxoids are relatively few and unimportant. Diphtheria Toxoid when used for children and adults is generally administered subcutaneously in two doses of 1 cc. each at intervals of 3 to 4 weeks. Infants under one year of age receive two injections of only one-half cc. in each dose. In the use of this as well as when all other prophylactic agents are employed to obtain a more lasting immunity to diphtheria, the Schick test and control are to be made three to four months or later after the (last) injection, to determine whether protection has been afforded. Toxoid (Formol-Toxoid) or Anatoxin has replaced the T-A Mixture for immunization as less injections are needed, the immunity is developed more quickly and a greater number of Schick positive persons become Schick negative. Diphtheria Toxoid is stable in its antigenic power, completely detoxified, not easily affected by temperature changes, but if it has become too clouded or turbid, it should not be used. The expiration date is two years after date of manufacture or date of issue.

Active immunization has resulted in a marked reduction in the mortality and incidence of diphtheria. Due to the appearance of a few Schick positive cases after immunization, it has been suggested that the Schick test be repeated once in 7 years (*N. Y. St. J. Med.*, *38*, 1227 (1938)).

REFINED DIPHTHERIA TOXOID (Alum Precipitated)

● It has been found that the addition of a small amount of a sterile solution of alum to a toxoid under suitable conditions will produce a complete precipitation. The supernatant fluid which is discarded contains as high as 80% of the protein in the original toxoid. The precipitate, after being washed, is resuspended in physiological saline solution (containing a preservative) and is marketed as a turbid suspension as indicated above. This product an alum precipitate of diphtheria toxoid in a highly purified state (free of serum albumin and of most of the albumin or protein contained in the original toxoid) is administered in a single dose treatment subcutaneously or intramuscularly (Glenny and Associates, *J. Path. and Bact.*, *29*, 38 (1926)). A Schick test is made two months after the injection. The toxoid is liberated but slowly from the precipitate, so that the antigenic stimulus is maintained for a longer period.

All Alum Precipitated Toxoids, being suspensions, must be shaken well before withdrawing the contents in the vial or container.

This insoluble suspension will be absorbed but slowly. The patient should be informed that a nodule or lump will appear after the injection and remain for several weeks. Not only is the pain of injection and reactions reduced to a minimum by this process of refinement, but present indications point to the fact that the degree of immunity secured from a single dose of Alum Precipitated Toxoid is higher than that secured from two doses of plain Toxoid or three doses of T-A Mixture (*J.A.M.A.*, *103*, 7, 227, 478 (1934)); (*Amer. J. Publ. Health*, *25*, 430 (1935)).

On the other hand some workers claim that the reactions produced are objectionable and Lai (*Chinese M. J.*, 1935, *49*:340) states that it does not give sufficient protection to all susceptible to diphtheria. Other precipitants are being tried. Precipitated toxoids are safe and highly antigenic. Inasmuch as they produce an immunity more readily than the original toxoids from which they were prepared, they may after additional experimentation replace all other antigens for active immunization against diphtheria.

Various conflicting opinions are available concerning the efficacy of the kind of toxoid and the number of doses to be used in immunization against diphtheria. An analysis of all of these opinions leads one to conclude that (1) Alum Precipitated Toxoid is superior to the Formolized Toxoid as an antigen; (2) If only one injection is to be given, the A. P. T. should be used; (3) If the F. T. is employed, at least 2 and preferably 3 injections are to be given; (4) However, whenever possible, the A. P. T. is to be the agent of choice and two injections are to be given. In order to eliminate the possibility of the occurrence of sterile abscesses which were reported when A. P. T. was first introduced, the following precautions should be observed: (a) Use completely detoxified toxin in its preparation; (b) Keep the total solid content consisting of bacillary protein, alum and other irritating substances as preservative in minimal amounts (toxins grown for periods longer than a week may contain increased quantities of bacillary protein); (c) The volume in the finished product should be 1 cc., not more and preferably not less; (d) Deep subcutaneous or intramuscular injections are to be advocated.

Toxoids wherever possible are replacing toxins as the agents to be used for the commercial production of antitoxins.

Other precipitants are being tried. Precipitated toxoids are safe, highly antigenic. Inasmuch as they produce an immunity more readily than the original toxoids from which they were prepared, they are replacing all other antigens for active immunization against diphtheria.

Workers from the laboratories of the New York City Department of Health have made many valuable contributions on diphtheria toxin (*J. Immunol.*, *16*, 421 (1929)) and on diphtheria toxoid, its preparation (*J. Immunol.*, *20*, 247 (1931); *28*, 215 (1935)) and antitoxic response of different kinds of preparations (*Am. J. Hyg.*, *29*, 89 (1939)). The following gives the method employed by this laboratory in the production of diphtheria toxoids.

PREPARATION OF DIPHTHERIA TOXOIDS

● "The diphtheria toxoids are prepared from high grade toxins. The toxins are made from bacto veal when a high grade regular toxoid is wanted or from fresh acetic acid veal when toxoid alum precipitate is the object.

Preparation of Bacto Veal Toxin— A purified previously tested for its efficiency bacto No. 2 dehydrated veal powder (veal + beef heart) prepared

by the Difco Laboratories is used. One pound of the powder per seven liters of distilled water is infused at 50° C. for ¾ hour. It is then boiled up for a few minutes and strained through cheese cloth. To the infusion, 2 per cent. of a tested proteose peptone and 0.5 per cent. of salt are added. The broth is adjusted to pH 7.3-7.4 by normal soda. It is then boiled up and filtered through cotton and a double layer of filter paper. The broth is put in specially devised pyrex toxin bottles (height 17¾ inch, width 7 inch, depth 4½ inch capacity 5400 cc.) in 800 cc. quantities per bottle. The sterilization is accomplished by the autoclave at 14-15 lbs. pressure for 45 minutes for the big bottles; ½ hour for smaller containers. The broth is prepared one week ahead so as to make sure of its sterility. For making the toxin the daily pellicles are grown twice a day and transferred first into the small flasks from which 18 hour pellicles are transplanted into the large toxin bottles. These are laid flat on their backs and incubated at 29°-30°C. for 7-8 days. Before inoculation 0.3 per cent. of sterile *maltose* and 1 per cent. of *sodium acetate* are added to each bottle. We are guided by the pH reaction for harvesting the toxin. This is usually about 8.4. The potency of the toxin is being tested each day by the Ramon flocculation test (from sample flask) so that by the time the toxin is ready to come out its potency and suitability for conversion into a toxoid is known. The potency is usually about 20-35 Lf per cc. or 1200-1500 MLD's per cc.

Fresh & Acetic Acid Veal Toxin—The muscles of the hind legs of young calves from 6-8 weeks old are denuded of fat and fascia. One pound of this lean veal is infused with one liter of distilled water to which 0.5 per cent. of glacial acetic acid is added. The infusion is brought to a boil slowly stirring constantly to avoid burning. It is then boiled for ½ hour and strained through cheese cloth. The subsequent treatment of the infusion for preparation of the broth is the same as for bacto veal. Before inoculation of the bottles, 0.3% of sterile maltose is added. The potency of toxin grown on fresh veal acetic broth though higher than grown on bacto veal is less uniform and varies with the season of the year. It may vary from 25-45 Lf per cc.

Preparation of D. Toxoid—To prepare a toxoid, the toxin of each bottle of toxin is thoroughly mixed with Merck's reagent formaldehyde neutral Assay 36%-38% HCHO. Before the formaldehyde is put in, a sample of the pooled toxin is taken for testing the L_+ or MLD potency of the toxin. The formalin toxin is filtered through paper pulp until crystal clear and put into 4 gallon bottles. These are incubated at 38°-40°C. for one month to six weeks. A sample of the pooled toxoid is then tested on guinea pigs for detoxification. The regulation from Washington is that 5 cc. given each of ten guinea pigs should not give any symptoms of diphtheria poisoning; such as induration, paralysis or death. In any such event, the toxoid is returned to the incubator for another week or two and again tested in guinea pigs. After detoxification it is Berkefelded and preserved with 1:10,000 merthiolate.

1—Bacto veal *toxoid* straight is given in school children.

2—Fresh veal *alum precipitate* is more widely used among young children. For adults, where a severe reaction from the injection is a handicap, as nurses, students, etc., the following preparation is used.

3—Preparation of *toxoid flocculi extract* is made by flocculating a toxoid with the estimated amount of a strong antitoxin. The toxoid is mixed with the antitoxin in quart bottles and the mixture poured into large

centrifuge tubes in which the flocculation occurs from 24-48 hours at room temperature. The contents are then centrifuged. The supernatant fluid is poured off and the flocculi washed with salt solution by centrifuging. The supernatant fluid is poured off and the flocculi dissolved in distilled water. The solution is usually concentrated to ¼ of its original volume. It is heated at 80° C. for 20 minutes and Berkefelded. The finished product is clear with a greatly diminished nitrogen content. It gives very little reaction, at the same time conferring a good immunity even with one dose of 1 cc. provided the product contains enough Lf units (usually 35 Lf per cc.).

Standardization of toxoids, toxoid alum precipitate and toxoid flocculi extract is very satisfactorily done by the Ramon flocculation test. The stronger the original toxin, the more Lf units in their toxoids and derivatives. The final test, however, is the immunity in the guinea pigs as expressed by survival from MLD's or antitoxin units in their blood.

For preparing *Toxoid alum precipitate* the percentage of alum for optimum precipitation of each lot of toxoid is first determined; this is usually between 1.5 to 2% of alum. The calculated amount of a 10% solution of potash alum is added to the toxoid for precipitation. The supernatant fluid is drawn off and the precipitate washed with salt solution to get rid of the alum. The supernatant fluid is drawn off again and the precipitate brought up with physiological salt solution to its original volume. Merthiolate 1:10,000 is added and the product tested for sterility. For testing of the Lf value one per cent. or more of sodium citrate is added to the toxoid alum precipitate at 40° C. for bringing it into solution."

STANDARDIZATION

● Various methods have been presented for determining the antigenic value of Toxoids. The *"Animal Test"* technique is used by some workers (See National Inst. of Health *"Minimum Requirements for Diphtheria Toxoid"*). In the latter, 300 Gm. guinea-pigs are inoculated with varying quantities of toxoid, then tested six weeks later for the resistance to different M. L. D.'s of toxin. 80% of such animals (using at least 10) when inoculated subcutaneously with the initial human dose of toxoid shall survive for at least ten days when later (at the expiration of six weeks) they are injected subcutaneously with 5 M. L. D.'s of a stable diphtheria toxin. Many workers always use this protection test as the final control. Another method is the:

"Binding of Antitoxin." When toxoid is added to a neutral mixture of toxin-antitoxin, it dissociates the latter mixture, unites with antitoxin and sets free a portion of toxin, so that a combination of toxoid and toxin-antitoxin will kill a guinea-pig into which it is injected. The stronger the toxoid (as expressed in antigenic units per cc. of preparation) the smaller will be the quantity necessary to affect this dissociation and the more rapid will be the death of the guinea-pig injected with the mixture. By determining the amount of toxoid necessary to dissociate an Lo mixture to render it toxic for the guinea-pig, the potency of the toxoid can be approximated.

"Ramon's Flocculation Test" (Ramon, G.: *Ann. Inst. Pasteur*, *45*, 291 (1931); *61*, 787 (1938)). This is the quickest method for determining the antigenic value of toxoid. When fresh diphtheria toxin and antitoxin are mixed *in vitro* in proportionate amounts, a precipitate or floccules are formed. A similar reaction will not occur with an excess or a too small

amount of antitoxin. A similar flocculation reaction will result if the toxin is replaced by toxoid. The reaction will be in the same degree as with the original fresh toxin from which the toxoid was prepared. This phenomena is an expression of neutralization and demonstrates the combining power of toxin or toxoid with antitoxin. Ramon suggested the term "antigenic unit" for the amount of toxin or toxoid equivalent to one unit of antitoxin as determined by the flocculation test. This is frequently spoken of as the Lf (Limit of Flocculation) dose. It has also been referred to as the "immuzing unit" (*U. I.*) (Schmidt, S., *Comp. rend. Sec. de Biol.*, 1923, *88*, 105). Ramon's flocculation method for standardizing diphtheria toxoid, (The Titration of Toxins and Antitoxins in "*New Knowledge of Bact. and Immun.*, Univ. of Chicago Press, 1928), yields a value corresponding to the antigenic units of the original toxin from which it is derived. Toxoids are standardized and generally prepared to contain at least 10 antigenic units in each cc. Some workers have questioned the value of noting on the label the flocculation or antigenic unit (Lf) as it may give a false security to the user. It has been shown that an increase or decrease in the Lf units does not alter in the same degree the protective action of the Toxoid. See also data under T-A Mixture.

The following are the '*Minimum Requirements for Diphtheria Toxoid*' revised and issued by the National Institute of Health as of Dec. 19, 1935:

1. The statement "No U. S. Standard of Potency" should not appear on the label.

2. No horse meat shall be used in preparing the broth from which the original toxin is made to be used in the preparation of diphtheria toxoid.

3. Diphtheria cultures for the production of toxin shall be incubated for not longer than ten days.

4. The original toxin from which diphtheria toxoid is made shall have an L dose of not more than 0.20 cc. or an M. L. D. of not more than 0.0025 cc.

5. Formalin used for detoxification shall conform to the requirements of the United States Pharmacopoeia and the smallest amount which will bring about complete detoxification shall be used, not exceeding 0.4 per cent.

6. Tests demonstrating detoxification shall be done as follows:

Five cubic centimeters of crude toxoid given subcutaneously in guinea pigs weighing 300 gms. shall cause no signs of diphtheria poisoning, including paralysis, at any time during a period of 30 days. A sufficient number of test animals shall be injected to insure that not less than four shall complete a given test, and animals dying during the 30-day period shall show no signs of diphtheria poisoning at autopsy.

7. Minimum antigenic requirements for crude toxoid shall be as follows:

At least ten guinea pigs weighing 270 to 320 grams shall each receive subcutaneously the initial human dose of crude toxoid. At the expiration of six weeks each of these pigs shall be injected subcutaneously with 5 M. L. D.'s of a stable diphtheria toxin, the toxicity of which shall be shown by the injection of 1 M. L. D. of toxin into normal guinea pigs weighing 250 grams at the same time. At least 80% of the test animals shall survive for 10 days.

8. Protocols of preliminary toxic and Antigenic and safety tests on each lot of crude toxoid intended for distribution shall be submitted to the National Institute of Health with 100 cc. of the final product and the lot held until released by report from the National Institute of Health.

9. Alum precipitated diphtheria toxoid shall bear the designation "Alum Precipitated" on the labels of the finished package.

10. Alum precipitated diphtheria toxoid shall be prepared only from crude toxoid meeting the requirements stated in No. 2, 3, 4, 5 and 6, and in addition shall meet the following antigenic requirements:

The human dose when administered subcutaneously to guinea pigs weighing 500 grams shall produce at least two units of antitoxin per cubic centimeter of blood serum at the end of four weeks. At least four guinea pigs shall be used for this test, but the blood serum from all guinea pigs may be pooled for the test for antitoxic content.

11. Alum used for precipitation shall comply with the requirements of the

United States Pharmacopoeia and the finished product shall contain not more than 20 milligrams of alum per human dose, the calculation being based on the total amount of alum added for precipitation.

12. For each lot of alum precipitated toxoid protocols showing detoxification as outlined in No. 6, and antigenicity as outlined in No. 10, shall be submitted to the National Institute of Health with 50 cc. of the final product, and the lot held until released for distribution by report from the National Institute of Health.

13. Phenoloid preservative shall not be used in diphtheria toxoid.

14. The maximum human dose of diphtheria toxoid shall not exceed one cubic centimeter.

15. The final container of alum precipitated toxoid shall contain not more than ten human doses.

16. In addition to the protocols outlined above, a designated information form shall be submitted for each lot of diphtheria toxoid before release by the National Institute of Health.

OTHER TERMS

● Under diphtheria test toxin, toxoid and antitoxin, the definition of the following values of toxin are given: M. L. D.; L_+; Lo; and Lf (or U. I.). Another value is the Lr (Limit of Reaction), which refers to the least amount of toxin which when mixed with 1 unit of antitoxin shows only a slight redness when injected intradermally into guinea-pigs (A. T. Glenny and K. Allen, *J. Path. and Bact.*, 1921, *24*:61). The Lo, L_+, and Lr of a stabilized toxin remain fairly constant, and any one of them may be (though in this country, the L_+ dose of toxin is the unit of measure) used in the standardization of antitoxins. The Lf and Lo doses of fresh toxin are alike if but little toxoid is present. When the latter is formed, the Lo dose increases in size but the Lf dose is not changed to any great extent. The Lf dose of a toxin expresses the combining power of a toxin and its corresponding antitoxin and therefore serves the purpose for determining the antigenic value of toxoids. The M. R. D. (Minimum Reacting Dose) is the least amount of toxin which when injected intradermally into a guinea pig will produce within 36 hours an area of hyperemia which is at least 5 mm. in diameter.

OTHER DIPHTHERIA PREPARATIONS EMPLOYED AS PROPHYLACTIC AGENTS

● In the British Pharmacopoeia, other than diphtheria toxoid preparations are included under the official Latin title *Toxinum Diphthericum Detoxicatum,* and they are also known as *Diphtheria Prophylactic.* The other chief types thus official in the British Pharmacopoeia are: *Diphtheria toxoid, alum precipitated; diphtheria toxin-antitoxin (T-A) mixture; diphtheria toxoid-antitoxin mixture,* in which the toxoid is sufficiently neutralized by antitoxin; *diphtheria toxin-antitoxin floccules* and *diphtheria toxoid-antitoxin floccules,* in which the floccules formed after flocculation are washed and then suspended in physiological saline solution. Diphtheria toxoid and all forms of diphtheria prophylactic must comply with tests as described in regulations under the Therapeutic Substances Act of 1925 to insure sterility, that the toxicity has been sufficiently reduced, and that the potency as immunizing antigen has been preserved. They are all administered subcutaneously and after a lapse of several months following the last injection (if two or more are to be given), a Schick test is performed to ascertain

if immunity has been established. A consideration of these other agents which are also employed as prophylactics to create a more lasting immunity against diphtheria follows.

DIPHTHERIA TOXIN-ANTITOXIN (T-A) MIXTURE

(For Establishing Active Immunity Against Diphtheria)

TOXIN-ANTITOXIN Mixture is a sterile, colorless liquid. It is prepared from diphtheria toxin which has been previously aged and standardized, then mixed with diphtheria antitoxin and finally diluted that the finished preparation contains a fairly large amount of toxin (1/10 L_+ dose) combined with a very minute quantity (0.08 unit) of antitoxin.

Although the earlier mixtures contained varying amounts of toxin even as high as 4 L_+ per human dose, it was found that smaller quantities of toxin would produce the same degree of immunity provided that the amount of free toxin per dose remained the same. As a result of the experimental work of Park and his co-workers in New York City, the Park-Banzhaf formula (*J.A.M.A.*, *63*, 859 (1914)), (*Am. J. Pub. Health*, *13*, 23 (1923)), was adopted in this country as being the most satisfactory. At present the mixtures in use are based upon this formula and contain 1/10 L_+ of diphtheria toxin per human dose but the amount of antitoxin has been reduced both actually and proportionately so that the mixtures conform to the regulation of the National Institute of Health, that:

"The ideal toxicity of diphtheria toxin-antitoxin mixture should be such that when 5 human doses of the sample (as ready for human use) are injected into each of five guinea-pigs weighing 300 grams two of these 5-dose pigs shall die acutely in less than 10 days and the other three shall die of diphtheria paralysis in from 15 to 35 days. Of five pigs each receiving one human dose, one should die of diphtheria paralysis in from 15 to 35 days, and the other four should be paralyzed, but survive. As permissible variations from this toxicity, guinea-pigs may be used weighing from 250 to 350 grams, the weights being scattered through this range for any set of guinea-pigs on the same dose. Of the 5 pigs on 5 human doses, from four to none may die acutely, more than 50 per cent of the remaining pigs dying of paralysis; and of the 5 pigs on 1 human dose, three to none may die of paralysis, some of the remainder surviving with paralysis. Pigs dying with other conditions than diphtheria poisoning (acute or paralytic) shall not be counted."

The usual precautionary measures for testing the toxicity, sterility, potency, etc., of all biological products must be observed in the marketing of this preparation. *T-A Mixture should not be kept at freezing temperatures, as the toxin is liberated by freezing thus increasing the toxicity of the product.* It should be stored preferably between 40° and 50°F. (5° to 10°C.). The expiration date is six months after date of manufacture or date of issue. T-A injections do not usually produce local or constitutional reactions in younger children or if they do occur, they are generally very slight. In older children, a redness or even swelling may appear at the site of injection and malaise and fever may occur, but in most instances these symptoms subside in 24 to 48 hours. Diphtheria antitoxin employed commonly is prepared from treated horses. The question was raised that if such antitoxin was used in making T-A mixture, the individual might become sensitized to horse serum to such a degree as to interfere later in life with an injection of any other remedial agent present in or prepared from horse serum. To remove and eliminate this remote possibility of sensitizing individuals to horse serum, the antitoxin in the T-A mixture is obtained from the blood of immunized sheep or goats. Neither of these two animals are ever or due to their size are likely ever to be used

for the production of any marketable therapeutic serum. In using diphtheria antitoxin coming from sheep or goats, the possibility of sensitizing to curative serums when using T-A mixture does not exist.

USES

● Active immunization against diphtheria is accomplished by using a dose of 1 cc. of the T-A mixture. Three injections, all the same strength, are given subcutaneously in the arm (at the insertion of the deltoid) at weekly or bi-weekly intervals. The development of an immunity frequently requires from eight to twelve months before the Schick test becomes negative. It is important that all children receiving T-A mixture should be Schick tested 12 months after the initial injection. From 10% to 15% may still show a positive Schick reaction. Such children require an additional series of (3) injections and are to be Schick tested several months after the completion of the second or each additional series of injections to determine whether an immunity has been created.

The preparation of a T-A Mixture, especially in which large amounts of antitoxin (as 3 units) are mixed with large (3 Lf) doses of toxin, so that the toxin is just underneutralized requires great care. The development of the "*Danysz Phenomenon*" must always be kept in mind. This effect considers the fact that the addition of the toxin to the antitoxin in separate portions, a definite period elapsing between the addition of the first and second fractions, results in much more toxin remaining free than when the total quantities are mixed at once.

In 1923, Park noted that if the *Standard* dose of 1 cc. toxin-antitoxin mixture is injected just beneath the skin of the anterior portion of the arm, a reaction is produced similar to the Schick reaction. This subcutaneous injection was substituted for the usual Schick test so that the first injection of T-A mixture served both as a test to determine susceptibility to and as the initital dose in an immunizing treatment against diphtheria. More pseudoreactions develop in older children and adults so that it is advisable to read the result of such an injection on the fifth, sixth or seventh day thus allowing ample time for pseudoreactions to subside. It must be remembered that unless the toxin-antitoxin mixture has the standard toxicity, this test is unreliable. Toxoid having no toxic action cannot be used in an initial injection to substitute for the Schick test.

FLOCCULES OR FLOCCULI

● When toxin and antitoxin are mixed together in proportionate amounts to effect a neutralization a precipitate is formed. A similar reaction will not develop with an excess or too small an amount of antitoxin (Ramon: *Compt. rend. Soc. de Biol.*, *86*, 661 (1922)). The reaction of precipitation can be greatly hastened if the test tubes containing such mixtures are placed in a water bath at a temperature of 50°C. to 55°C. It was also observed that flocculi are obtained from precipitation of toxoid and antitoxin. Such flocculi had the added advantage of doing away with the possible danger of the mixture becoming toxic. The immunizing value of toxoid-antitoxin flocculi is improved by heating at 80°C. as this temperature destroys the antitoxin. These precipitates or so-called Floccules or Flocculi (Toxin-antitoxin Flocculi or Toxoid-antitoxin Flocculi (*J. Bact. and Path.*, *30*, 587 (1927)) and even extracts of the latter (*Compt. rend. Soc. de Biol.*, *106*, 525 (1931)) have been used and are being employed successfully for

immunization against diphtheria without producing annoying or objectionable reactions. It is possible that products prepared along these lines may in the very near future be used as the most effective immunizing agents to afford a lasting protection against diphtheria.

DIPHTHERIA PROPHYLAXIS

● In considering artificial immunization against diphtheria, mention should be made of the two types of immunity or marked resistance which can be obtained or developed against this infection. Both of these are an acquired immunity and are either *Active* or *Passive.*

Active Immunity is due to the response of the individual's own body cells and tissues reacting and taking an active part to produce the specific immunity. The reaction is similar to that induced by an attack of the disease even though the symptoms of the disease itself are not pronounced or do not appear. In all instances of active immunity, the virus, or toxins, or causative agent of the disease itself, killed or modified, are introduced into the body of the animal. The reaction by the tissues of the latter is not instantaneous, so that active immunity is slow in the sense that it takes a length of time before the immunity is created. On the other hand, active immunity produces a more lasting protection, one which if it does not endure for life lasts at least for several years. The use of modified smallpox virus (so-called smallpox vaccine) creating an immunity against smallpox is an example of acquired active immunity.

Passive Immunity on the other hand is a protection created in an individual by introducing specific antibodies or protective substances without the body cells of the individual having reacted or contributed to the antibody production. Passive immunity is possible at a moment's notice and is quickly brought about by the mere introduction of the protecting substances, usually a serum containing antibodies. The use of diphtheria antitoxin in human beings is an example of passive immunity. Antibodies obtained from the serum of an actively immunized animal are passed or transferred to the individual to be protected. The latter plays but a passive part, receiving something possessing prophylactic properties which has been made outside of his body. Passive immunity lasts but for a short period of time as the antibodies are quickly used up.

When children or adults have been exposed to diphtheria they may be given immediate though temporary protection against the disease by the injection of 1000 units of diphtheria antitoxin. The passive immunity resulting from the latter lasts for a period of from 2 to 4 weeks at the utmost. Though it is known to all that diptheria antitoxin is of great value in the treatment of diphtheria where a large quantity of antibodies are required quickly to combat the invading extracellular toxins produced by the diphtheria bacilli, yet this same preparation is unsatisfactory as a product to employ for the purpose of developing or creating a *more lasting* or *permanent* immunity to this scourge. It is for this reason that efforts have been made to find materials which would be safe, would require simplicity in their use, and would possess definite advantages in producing an active and a more lasting immunity. What was wanted was a product and a technique which would be as effective against diphtheria as modified smallpox virus and vaccination are against smallpox. The introduction of satisfactory agents for active immunization and the production of a more lasting immunity to diphtheria have resulted after a long series of in-

vestigations in the presentation of the following products (*Med. Res. Council "System of Bact.,"* 1931, Vol. 6, 1931): *Diphtheria Toxin-antitoxin* (T-A) *Mixture; Diphtheria Toxoid-antitoxin Mixture; Diphtheria Toxoid* (Anatoxin, Ramon) and *Refined Diphtheria Toxoid* (Alum Precipitated); and *Toxin-antitoxin Floccules* or *Flocculi* and *Toxoid-antitoxin Floccules* or *Flocculi.* All of these preparations used for active immunization against diphtheria are recognized in the British Pharmacopeia under the title of *"Diphtheria Prophylactic"* or *"Toxinum Diphthericum Detoxicatum"*. The Toxin-antitoxin Mixture is not used to any great extent. Diphtheria Toxoid or Anatoxin (as the *Toxinum Diphthericum Detoxicatum* of the U. S. P.) and in particular Alum Precipitated Toxoid is the preparation most frequently used today the world over to create an active immunity against diphtheria. An important and valuable article on the procedure in the active immunization against diphtheria was issued by the Ministry of Health in Great Britain (Memo 170, Med., (1932)).

WHEN TO IMMUNIZE AGAINST DIPHTHERIA

● Age has long been recognized as an important factor and contributing influence to the contracting of diphtheria. It appears that newborn children are endowed with a certain amount of diphtheria antitoxin in the blood. This immunity in human infants is received from their mothers who if immune (and most adults are) transfer the antitoxin through the placental circulation and later probably during nursing. This transmitted immunity in the newborn may last up to twelve months, soon disappears, and is gradually reacquired naturally from the ages of ten to twelve and later. It is therefore exceptional to find infants of a few weeks suffering with diphtheria and children during the first six months of life are rarely attacked. The most susceptible period is between the ages of one to ten years. The dangerous period is between the ages of two to six. Most adults possess to some degree a natural immunity.

It must be remembered that a child under one year of age and even up to two years of age may give a negative Schick test, for reasons mentioned previously. Most of these children when tested at a later period however will give a positive Schick test. After four or five years of age, the reactions at this period of life (whether negative or positive) continue to be the same probably for life. Inasmuch as most children between one and six years of age yield positive Schick reactions, this test is generally omitted in practice so that if the young child was intimately exposed to a case of diphtheria, antitoxin is administered for immediate but temporary protection (passive immunization). Where there has been no exposure but a more lasting immunity is desired, T-A mixture or Diphtheria Toxoid and preferably Alum Precipitated Toxoid is administered (active immunization) without conducting the Schick test before initial injection. Schick tests are carried out 2 to 4 months after the last injection.

The tissues of infants and children are not so reactive as those of adults so that when immunizing infants, the choice of prophylactic generally is not of great importance from the standpoint of possible reactions. The toxoids and preferably the Alum Precipitated Toxoids are the agents most frequently used for immunization purposes. In the case of adolescents and adults however, severe and unpleasant reactions may occur especially after the injection of Toxoids. It may therefore be advisable to determine

the reactivity of the tissues of older individuals before immunization is practiced. The *"Moloney Test"* as introduced by Moloney and Frazer in Canada, is employed for this purpose. Injections of 0.2 cc. of a 1:100 or 1:200 dilution of toxoid are given intradermally. Well-marked local areas of erythema and induration indicate a "Moloney-positive" reaction and in turn a high degree of sensitivity. T-A Mixture or Toxoid-antitoxin Floccules are the most suitable agent for such positive reactors. If toxoid alone is only available, immunization should be carried out beginning with very small doses and gradually increasing the dosage.

At least two injections of toxoid, given 2 weeks apart, should be given. A Schick test is performed 2 to 4 months after the last injection and the latter dose repeated if the test is positive. After the first successful immunization, another Schick test is to be performed 3 years later. An injection of 1 cc. of toxoid is to be given if the test is positive or if it is not feasible to do the Schick test again, the single full dose of toxoid should be administered.

Parents should not delay safeguarding against diphtheria until the child reaches school age. Preschool protection is the best and safest. It is during the first three years of life that the susceptibility to diphtheria as well as the mortality is the greatest. The most suitable age for infants for immunization is six months in the country and nine months in the city or as soon thereafter as convenient. The preferred age of the infants in the country is earlier than in the city as parents in the country are more susceptible and less immune to diphtheria than parents in the city most of whom are immune.

INHALATION AND INUNCTION

● Experiments on immunization by inhalation and the rubbing in of ointments of anatoxins have and are being conducted. Guinea-pigs, mice and other laboratory animals were found to tolerate such treatment and immunity and protection were created. Similar prophylaxis was obtained experimentally with antitoxin and even some curative effect was observed with the latter when administered by inhalation (*Ann. inst. Pasteur, 52,* 690 (1934)). (*See also page 167*)

SERO-ANATOXIN THERAPY

● The administration of large quantities of diphtheria antitoxin does not prevent the production of active immunity by subsequent injections of specific anatoxin. There appears to be a synergism of practical importance between serotherapy and vaccinotherapy of diphtheria (*Presse med., 46,* 1542 (1938)). Ramon and Associates (*Rev. Immunol. 4,* 24 (1938)) report satisfactory results in the production of a high degree of immunity by this method. Other favorable reports are presented (*Rev. Immunol., 5,* 209 (1939); *Presse med., 47,* 797 (1939)).

VALUE OF DIPHTHERIA PROPHYLAXIS

● The efficacy of diphtheria prophylaxis is attested by the marked decline in the death rate of diphtheria wherever immunization procedures are in use. Furthermore, it must be remembered that diphtheria immunization is not compulsory, and therefore, is not used universally. However, voluntary cooperation has been satisfactory in many communities. The death rate in this country from diphtheria in 1900 to 1904 was 32.8 per 100,000 (*Met.*

Life Ins. Co. Stat. Bull., 19, 8 (1938)), while today the corresponding rate is 2.0. In a resume of 15 years of application of immunization against diphtheria, Ramon (*Ann. Inst. Past., 62,* 5 (1939)) presenting the results from all parts of France reveals the mortality in 1928 as 11.1 per 100,000 as compared to 2.7 in 1938 (last 8 months). In Belgium during the last ten years, 300,000 children were immunized and the efficacy of this procedure is recognized (*Presse Med., 47,* 637 (1939)). Statistical reports reveal similar findings on diphtheria in San Francisco (*Cal. & West. Med., 50,* 413 (1939)), in Chicago (*J. A. M. A., 112,* 1919 (1939)), in New York City (*N. Y. Med. Week, 18,* 6 (1939)), and in large cities throughout the United States (*J. A. M. A., 112,* 2043 (1939)).

XIX

TETANUS TOXOID

A TETANUS toxoid or anatoxin, made in the same manner as diphtheria toxoid, and a refined tetanus toxoid (alum precipitated) have been used successfully to actively immunize domestic animals against tetanus. Similar preparations have been used for the prophylactic immunization of humans (especially those in military service and others frequently exposed to tetanus), (*U. S. Naval Med. Bull. 36*, 524 (1938); *Royal Army Med. Corps J.*, May, 1938; *J. Allergy, 9*, 545 (1938); *J. Lab. Clin. Med., 23*, 903 (1938); *Presse med., 47*, 877 (1939); (*Am. J. Publ. Health, 29*, 334 (1939)).

Immunization with tetanus toxoid usually in two 1 cc. doses at intervals of 3 months either intramuscularly or by deep subcutaneous injection produces an active immunity lasting probably for many years. Its use eliminates the need for repeated antitoxin injections following injury, the possible subsequent serum reactions, and the possibility of sensitization to horse serum. Simultaneous administration of tetanus antitoxin and anatoxin constitutes a more satisfactory method of prophylaxis against tetanus. Such treatment prevents the (delayed) appearance of tetanus as is seen in cases treated only with antitoxin.

An interesting observation made by Pons (*Compt. rend. soc. biol., 129*, 209 (1938)) that tetanus toxin after transformation to the anatoxin loses its affinity for nervous tissue raises the question whether this loss of power of fixation in the nervous tissue does not explain the loss of toxicity.

PREPARATION

● Tetanus Toxoids are prepared as are Diphtheria Toxoids. The following minimum requirements issued by the National Institute of Health as of January 17, 1938, are to be observed:

1. The statement "No U. S. Standard of Potency" should not appear on the label.
2. Horse meat shall not be used in preparing the broth from which the original tetanus toxin is made.
3. Tetanus cultures for the production of toxin shall be incubated for not longer than 14 days.
4. The original toxin from which tetanus toxoid is made shall have a test dose of not more than 0.01 cc. or an M. L. D. for the guinea pig of not more than 0.0001 cc.
5. Formalin used for detoxification shall conform to the requirements of the United States Pharmacopoeia and the smallest amount which will bring about complete detoxification shall be used, not exceeding 0.4 per cent.
6. Tests demonstrating detoxification shall be done as follows:

Five cubic centimeters of crude toxoid given subcutaneously in guinea pigs weighing 300 grams shall cause no signs of spastic paralysis at any time

during a period of 21 days. A sufficient number of test animals shall be injected to insure that not less than four shall complete a given test.

7. Minimum antigenic requirements from crude tetanus toxoid shall be as follows:

At least ten guinea pigs weighing 300-400 grams shall receive, subcutaneously, the initial human dose of crude toxoid. At the expiration of six weeks each of these guinea pigs shall be injected with 10 MLDs of a stable tetanus toxin, the toxicity of which shall be shown at the same time by the injection of 1 MLD of toxin into normal guinea pigs weighing 350 grams. Eighty per cent of the test animals shall show no signs of spastic paralysis for a period of ten days.

8. Protocols of preliminary toxicity and Antigenic and safety tests on each lot of crude toxoid intended for distribution shall be submitted to the National Institute of Health with 100 cc. of the final product and the lot held until released by report from the National Institute of Health.

9. Alum precipitated tetanus toxoid shall bear the designation "Alum Precipitated" on the labels of the finished package.

10. Alum precipitated tetanus toxoid shall be prepared only from crude toxoid meeting the requirements stated in Nos. 2, 3, 4, 5 and 6, and in addition shall meet the following antigenic requirements:

The human dose when administered subcutaneously to guinea pigs weighing 500 grams shall produce at least two units of antitoxin per cubic centimeter of blood serum in not more than six weeks. At least four guinea pigs shall be used for this test. Aliquot parts of the blood serum from all guinea pigs may be pooled for the test for antitoxic content.

11. Alum used for precipitation shall comply with the requirements of the United States Pharmacopoeia and the finished product shall contain not more than 20 milligrams of alum per human dose, the calculation being based on the total amount of alum added for precipitation.

12. For each lot of alum precipitated toxoid protocols showing detoxification as outlined in No. 6, and antigenicity as outlined in No. 10 shall be submitted to the National Institute of Health with 50 cc. of the final product, and the lot held until released for distribution by report from the National Institute of Health.

13. Phenoloid preservative shall not be used in tetanus toxoid.

14. The maximum human dose of tetanus toxoid shall not exceed one cubic centimeter.

15. The final container of alum precipitated toxoid shall contain not more than 10 human doses.

DOSAGE AND ADMINISTRATION

● Two 0.5 cc. doses preferably of the Alum Precipitated Toxoid are given subcutaneously at an interval of 3 or more months. Immunity is established after a further interval of 3 to 6 months. An immunized person need not be given tetanus antitoxin when he has a tetanus exposure. He should however receive an additional dose of the alum toxoid. If an injury occurs during the time between the first and second injection or within two weeks after the second injection, it is advisable to administer the usual prophylactic dose of tetanus antitoxin.

Tetanus toxoids as other toxoids do not possess therapeutic value, are used only for active immunization, and are not to be used in the treatment of active cases of tetanus.

SERO-ANATOXIN THERAPY OF TETANUS

● As in the case of diphtheria, injections of tetanus antitoxin followed by the administration of the corresponding anatoxin seem to yield a high degree of immunity. Ramon and Associates (Rev. Immunol., *4*, 24 (1938)) obtained such findings experimentally in rabbits and clinically in humans. Other workers (Compt. rend. soc. biol., *125*, 711 (1937)) report upon the value of such therapy. In accordance with these findings, many workers advocate giving the first dose of tetanus (alum) toxoid at the same time as

tetanus antitoxin is given to subjects exposed to tetanus infection. They are administered at different sites. The second dose of alum toxoid is given two months later.

Combined Diphtheria Toxoid-Tetanus Toxoid, Alum Precipitated

THIS, a marketable preparation, consists of a combination of diphtheria toxoid and tetanus toxoid each having been precipitated with alum. The quantity of each ingredient in a single dose is the same as that present in a single dose of the individual marketed products. The individual toxoids are tested for toxicity prior to mixing. The combined alum precipitated toxoid is tested for toxicity after precipitation.

Two 0.5 cc. doses injected subcutaneously at intervals of 2 to 3 months, administered preferably in the region of the deltoid are employed. It is claimed that the immunity produced is frequently greater (in actual antitoxin content) for each of the specific diseases than results when immunization is practiced separately with the individual toxoids (*J. Lab. Clin. Med., 24,* 512 (1939)).

The potency of the preparation must be of such strength that when 500 Gm. guinea pigs are injected with one human dose, the blood serum of the animals should show at the end of 4 weeks the presence of at least 2 units of diphtheria antitoxin and 1 unit of tetanus antitoxin.

Toxoids by Intranasal Route

● Throughout this volume, many references are to be found concerning the intranasal administration of different biological products. Intranasal administration or nasal instillation is practical, simple in technique, usually free of untoward reactions. This procedure is most desirable especially if capable of producing by itself or at least maintaining in combination with other forms of therapy (with parenteral administration) a state of immunity. The use of various biological products intranasally and intranasal immunization specifically against diphtheria, tetanus, scarlet fever and bacillary dysentery have been reported (*Compt. rend. Soc. de biol., 96,* 762, 1205 (1927); *97,* 701 (1927); *Lancet, 1,* 491 (1938); *J. Bact. 36,* 322 (1938)). Further considerations of intranasal administration are given under *Pertussis Vaccine,* page *142.*

One of the large manufacturing biological laboratories is marketing various biologicals under the trade name of "*TOPAGENS.*" The latter are to be used for topical application intranasally (see also *Pertussis Topagen,* page 142). *Tetanus Toxoid Topagen* is available for nasal instillation. It is a highly antigenic purified alum precipitated toxoid concentrated to contain 25 Lf's per cc.

Several reports presented recently by different workers mention the value of Tetanus Toxoid immunization by the intranasal route. H. Gold of Chester, Pa. in a recent investigation (paper to be published) on active immunization against tetanus by the combined subcutaneous and intranasal routes concludes that this procedure is practical. He advocates as the first dose, at least one and preferably two subcutaneous injections of tetanus toxoid alum precipitated. This is followed by a course of nasal instillations of three daily treatments (0.1 cc. of Tetanus Toxoid Topagen in each nostril) repeated every 3 to 6 months. He states that active immunization can be maintained by these nasal instillations in advance of an injury. The antitoxin titer of individuals actively immunized by this method and followed every 3 to 6 months by a course of three daily instillations will remain constantly above the protective level.

XX

TOXINUM SCARLATINAE STREPTOCOCCICUM

Scarlet Fever Streptococcus Toxin

Toxin. Scarlat. Streptococ.—Scarlet Fever Streptococcic Toxin, Scarlet Fever Toxin for Immunization and for the Dick test.

"Scarlet Fever Streptococcus Toxin is a sterile solution in beef broth of certain products including a soluble toxin, resulting from the growth in the broth of suitable strains of hemolytic streptococci (*Streptococcus scarlatinæ*).

Description and physical properties—A transparent liquid having the color of the broth in which it is made (usually straw to brownish in color) and having a broth-like odor which is often altered somewhat by the antiseptic used as a preservative.

No horse blood or other foreign blood shall be added to the culture medium used for the preparation of the Toxin.

Scarlet Fever Streptococcus Toxin must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The potency of the Toxin shall be expressed in terms of the skin test dose, which is that quantity of toxin which will give positive reactions in all persons susceptible to scarlet fever and negative reactions in persons immune to scarlet fever, when injected intracutaneously.

The outside label must bear the name *Scarlet Fever Streptococcus Toxin,* the manufacturer's lot number of the Toxin, the name, address, and license number of the manufacturer, and a statement of the date beyond which the Toxin may not be expected to retain the potency prescribed by governmental authority.

Storage—Preserve Scarlet Fever Streptococcus Toxin at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose—For determining susceptibility (Dick Test), intracutaneous, 0.1 cc. of the dilution, representing one skin test dose.

For active immunization—For this purpose the Toxin shall be dispensed in a series of graduated doses of such potency and number that on the average, when the series has been injected hypodermically at proper intervals, into a toxin-susceptible individual, that individual will not react positively to the skin test dose of the Test Toxin, injected intracutaneously."

It has been known for a long period of time that certain hemolytic streptococci produce soluble toxic substances. In an extended series of investigations by many workers, culminating with the experimentations of Dick and Dick, convincing evidence was presented that there existed a close relationship between the streptocococcus and scarlet fever and that hemolytic streptococci isolated from scarlet fever cases elaborated a rash producing exotoxin. Based upon this specificity of the toxin, the Dicks and others have maintained that streptococci from scarlet fever are sufficiently different from other streptococci to be designated as a species, *Streptococcus scarlatinæ* (*J. A. M. A.*, *81*, 1166 (1923); *82*, 266, 301, 544, 1246; *83*, 84 (1924); *84*, 803, 1477 (1925); *93*, 1784 (1929); *98*, 1436 (1932)). On the other hand, it is known that the various strains of streptococci from scarlet fever differ in agglutination and even in antigenic properties. It is therefore apparent that streptococci cannot be differentiated merely upon the basis of specific toxin production and many workers are of the opinion that the designation *Streptococcus scarlatinæ* as a distinct species is not warranted at present. More recently (*Sci. News Letter*, Jan. 8, 1938), Broadhurst presented evidence to show that a virus, as was formerly held by others, is actually the cause of Scarlet Fever.

Although it is not always possible by present methods to definitely distinguish hemolytic streptococci found in cases of scarlet fever from those associated with other infections the practical value of this streptococcus toxin from scarlet fever cases in the intracutaneous test for susceptibility (Dick Test), for the active immunization (preventive treatment) against scarlet fever and for the preparation of antitoxin of value in the treatment (passive immunization) of scarlet fever has apparently been demonstrated. In the preparation of this toxin, the selection of one or more strains of streptococci from scarlet fever cases for toxin production must be attended with care. The strain or strains employed must produce a highly potent toxin and should possess broad antigenic activity. The Dochez NY 5, Dick II and Williams Sc. 2 strains have been found most satisfactory. Some workers prepare the finished Dick Toxins by mixing the filtrates of cultures of several strains.

The strain selected or standard strains which are obtainable (and approved by the Scarlet Fever Committee, Inc. and the National Institute of Health) are grown for seven days at 37°C. in dextrose-beef-infusion-peptone or Douglas's tryptic digest broths, 0.5% phenol or other suitable preservative in proper concentration is added and the culture is filtered through cotton, soft filtering paper and then through a Berkefeld filter to free it of bacteria. Exposure to light is avoided during the process of manufacture as well as during storage. Sterility, safety and potency tests are conducted. This toxin is marketed in a diluted form (each 0.1 cc. containing one skin test dose) and generally labeled Scarlet Fever Streptococcus Toxin for the Dick Test (or for Determining Susceptibility to Scarlet Fever). In the case of the latter the parent toxin is diluted with a properly buffered isotonic diluent (as 0.05% to KH_2PO_4; 1% $Na_2HPO_4.12H_2O$; 0.6% NaCl, and 0.5% phenol in neutral distilled water) adjusted to and kept at a pH of 7.5. The diluted toxin is stored between 2°C. and 5°C. (avoid freezing) for a period of 30 days immediately following its dilution and its titer is then determined. This storage period stabilizes the loss of demonstrable antigen which occurs over a brief period following dilution. A boric acid borate buffer solution (B. B. S.) is also recommended as a diluent (*Brit. Med. J.*, *II*, 700 (1938)). The other marketable form in a

series of graduated doses of suitable strength and number is generally labelled *Scarlet Fever Streptococcus Toxin* (For (Prophylactic or Active) Immunization). The expiration date for these marketable products is six months after date of manufacture or date of issue for diluted toxin and one year after date of manufacture for undiluted toxin and storage is in the cold (between 2°C. and 10°C.). The following are the *"Minimum Requirements for Scarlet Fever Streptococcus Toxin for the Dick Test and for Immunization,"* authorized by the National Institute of Health as of January 1, 1937:

1. The statement "No U. S. standard of potency" need not appear on the labels.

2. The source of the strains of hemolytic streptococci used in the preparation of the toxin must be stated in the application for a license. No strain of the hemolytic streptococcus may be used unless it has been shown that the erythrogenic toxin produced is neutralized by convalescent scarlet fever serum. The above information in regard to the source and specificity of the organisms used shall be submitted in reference to any new strains of the organism which it may be desired to introduce after a license has been procured. Subcultures of such new strains shall be submitted to the National Institute of Health with the above information.

3. Smears and subcultures in broth and on blood agar shall be made from each toxic culture from which it is intended to harvest the toxin, stained by Gram's method, and examined morphologically and culturally for evidence of contamination. Containers showing evidence of contamination shall be discarded.

4. No horse blood and not more than 1% of any other blood may be added to the medium prepared for toxin production.

5. The toxic culture shall be filtered through a filter of such a grade that the filtrate is rendered sterile.

6. The potency of the new toxin shall be determined by the character of the skin reactions resulting from the intracutaneous injection of suitable dilutions in 0.1 cc. amounts on suitable test individuals (the designation "individual" is defined in its broader biologic sense to include man or any other suitable test animal) that give positive reactions to the correct dilution of the control toxin supplied by the National Institute of Health and which will also give negative reactions on test individuals giving negative reactions to the control toxin. The reactions caused by the new toxin on different test individuals of varying degrees of susceptibility shall be comparable in every way to the reactions produced by the control toxin.

7. Protocols showing the following in regard to each new batch of toxin shall be sent to the National Institute of Health: (1) The reactions caused by the new toxin and the control toxin on at least 20 individuals who give positive reactions to the control toxin and at least ten individuals who are negative to the control toxin. (The 20 positive tests should include strongly, moderately and slightly positive reactions). (2) The intensity of color and presence or absence of swelling in the area reacting to the test dose of the new toxin as compared to the reaction caused by the control toxin supplied by the National Institute of Health. (3) The vertical and horizontal diameters (expressed in mm.) of the reactions produced by each batch of new toxin and similar diameters of the reactions produced by the control toxin injected in like volumes on the same individuals at the same time. (4) The potency of the new toxin based upon the foregoing tests.

A reasonable quantity of each new batch of toxin whether for susceptibility test or for immunization shall be submitted to the National Institute of Health. The product shall be withheld from distribution until passed by the National Institute of Health. No toxin shall be distributed unless the undiluted toxin be of sufficient strength to permit a dilution of 1 to 1000 for skin test use.

8. The number of test doses in the contents of each final container of toxin for susceptibility test shall appear on the label. The volume of solution to be given as a test dose shall also be stated on the label.

9. The number of skin test doses in each dose of the immunizing material shall appear on the label. The volume of the immunizing solution to be given in each dose shall be stated on the label.

10. Undiluted toxin may be given a dating of five years from the date of manufacture (the last date of satisfactorily passing a potency test). A renewal of the dating will be allowed for an additional five-year period on the presentation of a suitable protocol such as is designated under paragraph 6 above.

11. Diluted scarlet fever streptococcus toxin may be given a dating of six months from date of manufacture or issue. If the date of issue is used the product shall be kept undiluted between the date of manufacture and the date of issue.

12. The general requirements for the preparation of biologic products shall be in force in the production of Scarlet Fever Streptococcus Toxin insofar as those requirements are not in conflict with these tentative specifications.

In addition to the control exercised by the National Institute of Health, samples representing each lot of Scarlet Fever Streptococcus Toxin and other scarlet fever streptococcus products are submitted to the Scarlet Fever Committee, Inc., which administers the patents granted to Drs. G. F. and G. H. Dick. This committee independently standardizes and tests clinically the finished products before they are released for distribution.

The so-called scarlet fever streptococcus toxin is relatively heat stabile. The fresh filtrate may reveal an increase in toxicity at the beginning when stored but after a few weeks in the refrigerator the toxicity is stabilized and then diminishes very slowly when at 5°C. to 10°C. being much more rapid at room temperature. Alkali causes a rapid loss of toxicity especially of diluted toxin. Even the small amount of alkali as may be present in some glassware and vials or ampules and rubber stoppers, or that may get in by boiling syringes in alkaline tap water instead of *distilled water* will cause rapid deterioration of the toxin. Attempts to sterilize syringes and needles with alcohol are to be avoided as the alcohol precipitates the toxin.

STANDARDIZATION

● Scarlet fever toxin is not toxic to lower animals. Its action is most conspicuous upon human beings. When the latter are injected intracutaneously with small amounts of the toxin, a local reaction is produced within 18 to 24 hours, and is revealed by the presence of a bright red, swollen area varying in dimensions according to the amount of toxin injected and the susceptibility of the subject. This is the basis of the Dick test and the basis for the technique employed to determine the potency of this toxin. The unit of scarlet fever toxin is the skin-test dose (S. T. D.). "The skin-test dose is the smallest amount of toxin which when injected intracutaneously into persons known to be susceptible to the toxin will induce a reaction equal to that induced on the same persons at the same time by the injection of a skin-test dose of the standard scarlet fever toxin supplied by the National Institute of Health." The dose is contained in 0.1 cc. and a positive-test is a circumscribed area of redness and slight infiltration which measures at least 1 centimeter in diameter. The readings are made on a group of susceptible children (10 or 20 by preference) in a bright light in from 20 to 24 hours after injection and compared with the reaction induced by the standard toxin. The average of the smallest dilution of the toxin tested giving a reaction of 1 cm. and equal to that induced by the standard toxin is recorded as the skin-test dose. Toxins possessing a potency of 40,000 skin-test doses per cc. or greater are obtainable and are to be preferred to the low potency toxins. The skin-test dose of the present standard toxin is 1/30,000 cc. In commercial laboratories, a common practice is to make preliminary standardization tests on goats or

other animals, employing multiples of the human skin-test dose. White-skinned goats, grade or pure-bred Saanen, and from one and one-half to four years of age are preferred. The same animal may be employed repeatedly providing the reactions induced by the standard toxin are definite and controls with heated toxin, boiled for at least two hours and with uninoculated toxin broth are satisfactory. At least two weeks are allowed between tests. Final tests of the marketable toxin are always made on human subjects.

Recent investigations have revealed the possibility of a flocculation test (scarlet fever toxin flocculation with antitoxin) as a means of guidance in the production of crude scarlet fever toxin of high potency. The toxin is prepared in a semi-synthetic culture medium (*Am. J. Publ. Health, 29,* 139 (1939)).

USES

As A Diagnostic Agent in Dick Test

● Scarlet Fever Streptococcus Toxin is used in the Dick Test, an intracutaneous test performed to determine susceptibility or immunity to scarlet fever. For this purpose, the toxin is properly diluted with suitable diluent so that a skin-test dose is contained in each 0.1 cc. of marketable preparation. The injections are to be measured accurately, are given intradermally generally on the flexor surface of the forearm after the latter is asepticized, and reactions must be read preferably in a bright light in from 20 to not later than 24 hours after the injection. For careful work a control test to rule out pseudo-reactions may be made by conducting the test in the same manner as the original and employing as a control the same amount of toxin but which was heated to boiling for at least two hours. In routine work especially for ordinary testing in children, a control test employing heated toxin is not necessary when performing the Dick Test, as pseudo reactions are not commonly observed. Reactions produced in a Dick Test reveal all gradations of redness and inflammation and these differences in intensity correspond to different degrees of susceptibility to scarlet fever. In a correctly made and properly interpreted test using standardized material, a positive reaction is said to indicate susceptibility to scarlet fever while a negative reaction is considered as an indication that there exists sufficient antitoxic immunity to be protective against this disease. The following designations are used by some workers in recording the readings of a Dick Test: (a) Negative—erythematous patch not apparent or only slight and less than 1 cm. in diameter; (b) Slight Positive—red area, 1 to 2 cm. in diameter; (c) Positive—red area, 2 to 3 cm., with slight swelling and tenderness; (d) Strong Positive—red area, over 3 cm. in diameter, swollen and tender.

Active Immunization

● Scarlet Fever Streptococcus Toxin is not to be used in the treatment of scarlet fever patients but it is of value in the preventive treatment of this disease. The administration of suitable amounts of the toxin as a prophylactic will cause the Dick test to become negative in most humans. Upon this basis accepting the Dick test as an evidence of immunity, techniques have been developed for the active immunization of individuals against scarlet fever. It is becoming increasingly common to actively immunize children (in institutions, schools, etc.) and medical and nursing staffs as well as everyone liable to be exposed to scarlet fever so as to

afford a protection which will endure. Dick and others have advised against its use as an immunizing agent for those recently exposed and who are Dick positive unless a culture of the nose and throat reveal the absence of hemolytic streptococci, and further exposure can be avoided during the course of immunization. The toxin is injected subcutaneously in the deltoid region, the dosage varying depending upon different workers, but in all instances the doses are being increased from year to year. Some recommend giving the injections intramuscularly. The reaction to the Dick Test appears to be a reliable guide for the determination of dosage (*Wisconsin M. J.*, *37*, 797 and 804 (1938), (*Can. Pub. Health J.*, *29*, 439 (1938)). A common practice by many is to give a series of five injections at intervals of 5-to-7 days in the following schedule of dosage: (1) 500 S. T. D.; (2) 2000 S. T. D.; (3) 8000 S. T. D.; (4) 25,000 S. T. D.; (5) 80,000 to 100,000 S. T. D. The first four doses are contained in volumes of one cc. and the fifth dose is present in a volume of 2 cc. Children under 1½ years of age receive one half the usual dose. A Dick Test is performed two to three weeks after the last injection of toxin is administered. If still positive the last immunizing dose is repeated until a negative test is obtained. Intradermal immunization (*J. Clin. Investig.*, *16*, 671 (1937); (*J. Infect. Dis.*, *62*, 83 (1938); (*Ill. M. J.*, *74*, 248 (1938)) has recently been recommended as not producing the unpleasant local and general reactions which often follow subcutaneous immunization.

Scarlet Fever Toxin is made without and therefore does not contain any serum. The filtrate does however contain in addition to the soluble exotoxin, foreign protein due to a considerable amount of waste products of the growth of the organisms, some bacterial proteins and constituents of the medium. Local reactions nearly always occur at the site of injection. General reactions though mild may occur, especially in the early injections. It appears that giving the patient a mild purgative (milk of magnesia) the night before, administering the toxin in the late afternoon and partaking in a light diet tend to allay or reduce to a minimum any discomfort due to reactions. The results of prophylactic immunization as reported by workers have been variable. In analyzing some of these findings, errors are found especially in the fact that in many cases immunization was not carried through until a negative Dick test was obtained in those originally positive. In the hands of careful workers there is however available substantial evidence that prophylactic immunization properly carried out will confer an adequate protection and is a valuable method for the reduction of the incidence of scarlet fever (*J. Indiana M. A.*, *30*, 332 (1937)). The duration as well as the degree of the active immunity depends upon the amount of toxin injected. Sufficient time has not elapsed to allow the formation of definite conclusions. There is evidence however to show that in the great majority of cases immunization if properly carried out is complete for at least six years, so that if a child is treated and thus protected during the earlier years of life when the risk of the infection is greater and actual physical resistance is not as great as in the later years, the child can be bridged over a dangerous period until the time when the possibility of contracting the disease is almost at a minimum.

It is of sufficient importance to note and caution workers against the use of Scarlet Fever Streptococcus Toxin as a therapeutic agent in the treatment of scarlet fever patients.

SCARLET FEVER TOXOID

● Formaldehyde added to Scarlet Fever Toxin will reduce or remove the toxicity of the latter, leaving most of the antigenic property intact. Such toxoids have been prepared in the usual way by adding 0.3 to 0.4 per cent of formaldehyde solution to a standardized toxic filtrate of scarlet fever streptococcus cultures and this mixture is allowed to incubate at 37° C. for eight weeks or until markedly detoxified as revealed by intradermal injections of dilutions. The dosage of the finished toxoid is in terms of S.T.D. of raw toxin equivalence, the latter having been standardized before detoxification. Especially since the success of the use of diphtheria toxoid, similar work has been carried on experimentally with the use of toxoids for active immunization against scarlet fever. Many workers have reported that individuals tolerate much larger doses of scarlet fever streptococcus toxoid so that it appears probable that the number of injections for active immunization may be reduced. Reactions where produced are apparently very mild though some lots of material give severe reactions, (*Chinese Med. J. 53,* 427 (1938)). Perhaps with the development of a suitable toxoid or other suitable product, one highly antigenic and non-toxic which will require three or preferably less than three injections, mass or universal immunization against scarlet fever will come into more general favor. Many clinicians are reluctant and many patients refuse to use the scarlet fever streptococcus toxin at present as at least five immunizing doses are needed. This means more discomfort and greater expense. Experimentation with scarlet fever toxoids are being carried on with the hope that a product may be developed which will be more like diphtheria toxoid in its almost complete freedom from reactions and in the number of injections required.

Some workers are of the opinion that it is desirable to produce antibacterial as well as antitoxic immunity by vaccination against scarlet fever with vaccines and using not only the toxin or toxins or toxoids, but the streptococcus itself. This possibility is suggested by the results of experiments on the active immunization of rabbits, (*Arch. Pediat., 56,* 74 (1939)).

STAPHYLOCOCCUS TOXOID

STAPHYLOCOCCUS Toxoid as a marketable preparation has found extensive use in the treatment of localized staphylococcus infections. Much work has been done recently on the preparation, effects and value of staphylococcus toxin, toxoid and staphylococcus antitoxin, the latter being advocated in the treatment of persons suffering from staphylococcus toxemia or septicemia (*J.A.M.A.*, 1934, *100:*1007; April 20, 1421); (*Compt. rend. soc. biol.*, 1934, *117:*717; 1935, *118:*212, 1062); and (*Lancet*, 1934, *226:*1054; 1935, Feb. 9, 303 and 306). The marketable Staphylococcus Toxoids are labeled with the number of necrotizing doses of toxin per cc. (*Quarterly Bulletin, Health Org.*, Leag. of Nat., 1934), a necrotizing or dermonecrotizing dose of staphylococcus toxin being the least amount which on intradermal injection into the skin of a susceptible rabbit produces an area of erythema with a central necrosis of at least 5 x 5 mm. in diameter.

PREPARATION

● Suitable strains of staphylococcus capable of producing a potent exo-

toxin having characteristic destructive properties against living cells and tissues are selected. The toxoid is prepared by treating the toxin with formaldehyde in a procedure as used in the preparation of other toxoids (*J. Path. & Bact.*, *35*, 199 (1932)). The finished marketable preparation is a clear straw-colored fluid and standardized in two strengths so that each cc. contains an amount of toxoid derived from at least 100 or 1,000 necrotizing doses of toxin (see under Staphylococcus Antitoxin). It is Berkefeld filtered, tested for sterility and toxicity by the usual tests and a suitable preservative is added (usually merthiolate 1:10,000) (*J. Immunol.*, *29*, 209 (1935)). Toxoid which appears cloudy or turbid should not be employed.

Johlin (*Proc. Soc. Exptl. Biol. Med. 41*, 135 (1939)) presents a technique in which formal detoxification of staphylococcus toxin is accelerated. Staphylococcus toxin is completely detoxified by emulsification with chloroform in the presence of 0.25% formaldehyde in about 2 hours, and almost equally as fast when emulsified with oxygen and nitrogen. Detoxification requires days if the toxin is allowed to stand with formaldehyde without emulsification.

USE AND DOSAGE

● Staphylococcus toxoid induces the production of staphylococcus antitoxin in the blood of those immunized. It has been reported as a valuable agent in the prophylaxis and treatment of boils or carbuncles, pustular acne, staphylococcal abscesses of soft tissue and bone, and in other staphylococcal pyodermas and localized pyogenic processes due to Staph. aureus or albus. It is not to be used in general blood stream infections (*Brit. Med. J.*, *I*, 98, II, 335 (1937)). Injections are given subcutaneously or intramuscularly at intervals of 4 to 7 days. The scheme of dosage is to start with a volume in the first injection containing 20 to 40 necrotizing doses. The dose is doubled in subsequent injections depending upon the appearance of reactions. If the latter prevail, the dosage is reduced. From 6 to 10 injections are given and this course of treatment should be instituted even though the infection appears to have cleared up.

In a recent report, French workers, (*Presse med. 47*, 243 (1939)), using staphylococcus anatoxin in over 2000 cases of staphylococcus infections conclude that this is a most valuable agent. Their conclusion is supported by the experience of other investigators in France, in the colonies and in other countries. They claim that failures reported are due to the use of anatoxin with too weak antigenic power, or in too small doses. The value of the anatoxin is especially marked in furunculosis (cures were obtained in 90% of 1000 cases treated).

STAPHYLOCOCCUS ALUM PRECIPITATED TOXOID

FARRELL and Kitching (*J. Immunol. 34*, 51 (1938)) and other workers have recommended the purification staphylococcus toxoid by alum precipitation. The antigenic potency of suspensions of alum precipitated toxoid was found to be greater, especially in the fact that a much earlier response was obtained.

STAPHYLOCOCCUS AMBOTOXOID

(Staphylococcus Bacteriophage Toxoid)

THIS preparation is said to contain the exotoxic and endotoxic principles of the staphylococcus (*Amer. J. Publ. Health, 26,* 1001 (1936)). It is prepared by reinoculating a potent staphylococcus toxin with pooled cultures of staphylococci and with an adapted staphylococcus bacteriophage. After lysis, the bacteriophage toxin is converted into toxoid in the manner as is used in preparing plain staphylococcus toxoid. Tests for toxicity and potency are conducted. It is marketed in containers similar to staphylococcus toxoid and its dosage, method of administration and other considerations are as for the latter. It is reported that as an antigen it yields antitoxin contents consistently higher than that resulting from the immunization by staphylococcus toxoid or bacteriophage solution.

OTHER TOXOIDS

Investigators have been and are experimenting with all toxins and toxic filtrates produced by bacteria with the thought of obtaining detoxified toxins which will display antigenic properties. The more important ones were detailed previously, while in few instances only mention was made (and references given) of those which have not as yet received wide acclaim.

A new *meningotoxoid* was recently presented by Oz (*Science, 89,* 588 (1939)). Broth cultures of meningococcus at pH 7.7 containing 2% dextrose were incubated at 37°C. for twelve to fifteen days and filtered. The filtrate was treated with 3 to 4% of formaldehyde and incubated at 40°C. for forty-five days. 1.5% of alum was added and the preparation was washed and finally resuspended in saline. Antibody formation occurred in human subjects after consecutive injections of 0.5, 1.0 and 1.5 cc. of the non-precipitated toxoid or 1.0 cc. of the precipitated toxoid.

XXI

TUBERCULINUM PRISTINUM

Old Tuberculin

"Tuberculin is a sterile solution in a special liquid culture medium of the soluble products of growth of the tubercle bacillus (*Mycobacterium tuberculosis*) and should contain about 50 per cent of glycerin.

Description and Physical Properties

A clear, brownish liquid, readily soluble in water and having a characteristic odor. Tuberculin must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

Tuberculin must be capable of effecting a general and local response in tuberculous guinea-pigs.

The outside label must bear the name *Tuberculin Old,* the manufacturer's lot number of the Tuberculin, the name, address, and license number of the manufacturer, and a statement of the date beyond which the Tuberculin may not be expected to retain the potency prescribed by governmental authority.

Storage

Preserve Tuberculin at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

By intracutaneous injection, 0.001 cc."

Large shallow flasks containing 5 percent. glycerin alkaline broth are inoculated with a culture of a standard human strain of the tubercle bacillus (*Mycobacterium tuberculosis, hominis*). The flasks or bottles are capped with sterile tinfoil and placed in an incubator at 37° C. where they are kept undisturbed for from six to nine weeks depending upon the rate of growth and upon the appearance of a pellicle which first covers the surface of the medium and finally sinks to the bottom. Each container is then agitated well until the pellicle is suspended uniformly throughout the culture medium and the contents are subjected to a current of steam at 100° C. until the mixture is reduced to one-tenth of the original volume. The material is filtered through paper pulp, cresol to 0.3% or other suitable preservative to proper concentration is added and final filtration is

conducted through Berkefeld filters. Sterility, toxicity, safety and potency tests are conducted in accordance with the practice for other biological products. This product retains its activity for an indefinite period of time, though the expiration date generally employed is five years after date of manufacture or date of issue. As a matter of precaution, it should be kept in a cool, dark place.

Koch's Old Tuberculin is a heated 50 per cent glycerin solution of the soluble products of the tubercle bacilli containing the original elements of the culture fluid. Many attempts have been made to adopt some uniform and simple methods for determining the efficiency of this as well as of the other Tuberculins mentioned later. A satisfactory and reliable method has not as yet been made available. Among the several methods proposed are to be found the following: (a) the M.L.D. or minimal lethal or killing dose for tuberculous guinea-pigs when injected subcutaneously; usually 0.25 to 0.5 cc. of the undiluted tuberculin injected subcutaneously will kill a tuberculous guinea-pig in twenty-four to forty-eight hours; (b) the skin-reacting dose in such pigs; (c) titration, employing the skin of a tuberculous human; and, (d) the spermatocyte inhibition test of Long where tuberculous pigs are given intratesticular injections with varying amounts of tuberculin and the minimal amount which will cause a complete inhibition of spermatogenesis (this amount then known as the "spermatocyte unit") is noted. The marketable preparations are generally compared with standard samples of known toxicity and potency to note the dose necessary to produce the same effect as a definite amount of such efficient standard product.

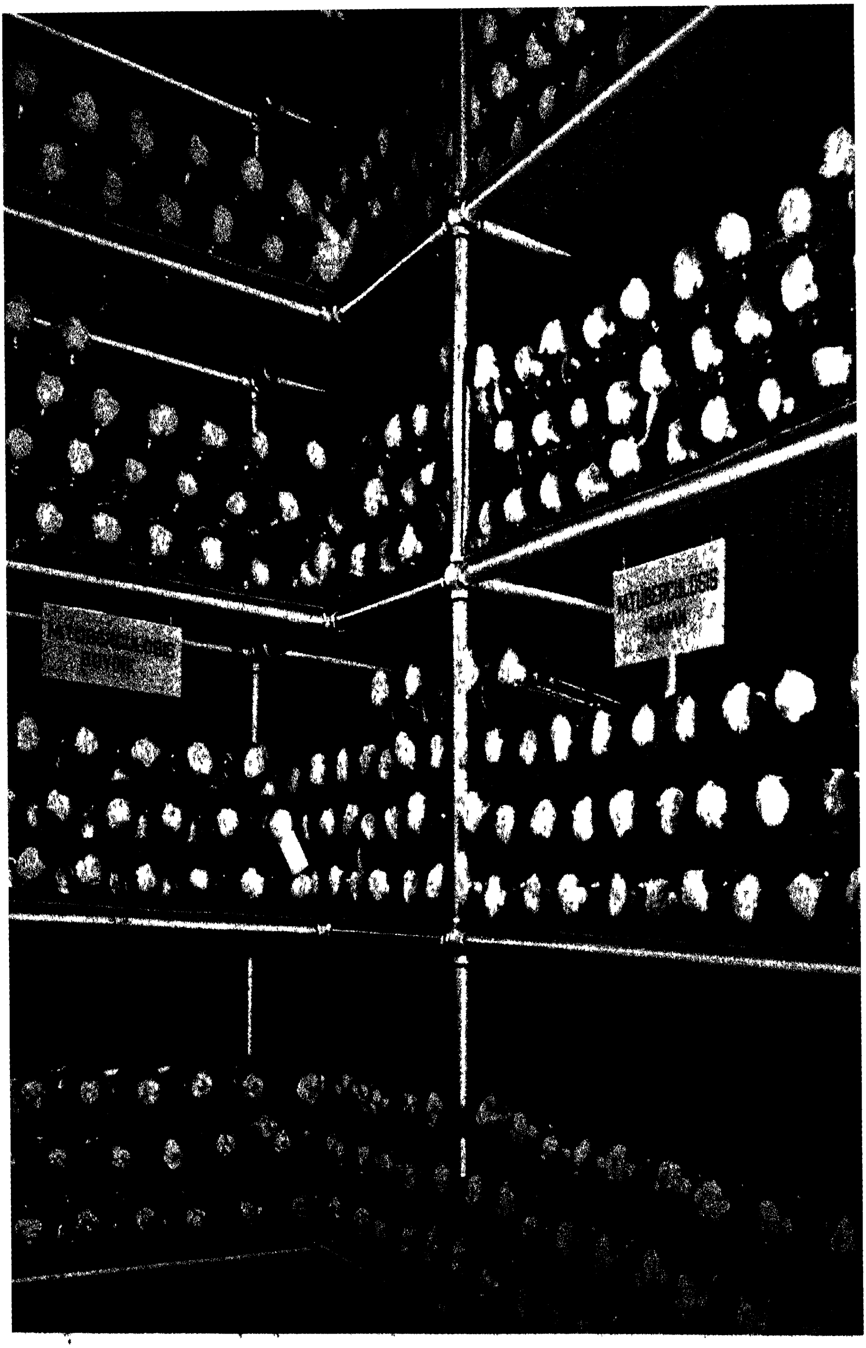
USES

● Koch's Old Tuberculin was introduced originally by Koch as a therapeutic agent.

Its use as a curative agent in tuberculosis is not without danger. At present, the use of this and other tuberculins in the treatment of tuberculosis is only advocated to be employed by those practitioners who have a thorough understanding of its limitations and possibilities. The tendency has been toward small doses, to be used in early conditions, not advanced cases, and in those in whom the tuberculous process is not a very acute one. Doses of from one hundred-millionth (0.00000001) to one millionth (0.000001) cc. may be employed as the initial dose and not more than two subcutaneous injections (preferably in the back at the lower angle of the scapula) are given each week. The dosage is gradually and progressively increased over as long a period of time as is needed to avoid reactions. Larger or excessive doses at more frequent intervals and intramuscular and intravenous injections are to be avoided. If reactions develop, the next injection is to be given only after all symptoms of such reactions have completely disappeared. The length of the course of tuberculin treatment varies considerably depending upon individual circumstances. Many workers stop further injections when they have reached the dose of 0.1 cc. of the undiluted tuberculin though some continue until the 1 cc. dose is reached. As a safe rule it can be said that injections are to be given as long as the clinical symptoms continue to improve and such time may vary from four months to from one to two years. Favorable reports are recorded in its use in different forms of tuberculosis, especially that of the urinary tract and in uveal tuberculosis (*J. M. Soc. New Jers.*, *35*, 138 (1938)). The use of tuberculin as a supplement to sanatorium and collapse therapy has been

Transplanting a culture of the tubercle bacillus.





Section of incubator showing the culture bottles for growing tubercule bacilli for the production of Tuberculin.

advocated especially in those cases which fail to improve under sanatorium and collapse therapy alone, (*Am. Rev. Tub.*, *39*, 318 (1939)).

Tuberculin should never be sprayed out of a syringe or thrown about carelessly as serious reactions may be produced in those exposed to these preparations under such conditions.

VALUE

● Koch's Old Tuberculin is of greatest value as an agent to be employed as an aid in the diagnosis of tuberculosis in man and animals. A human being or bovine animal revealing a significant sensitivity to tuberculin (application of tuberculin to the skin or mucous membranes produces a more or less severe inflammatory reaction at the point of application) is generally considered as having a tuberculous focus in his system (though the latter may not necessarily be active). It does not reveal the extent or activity of an existing lesion. Sensitivity to tuberculin may be absent in acute miliary and advanced generalized tuberculosis and in some acute infectious diseases. Of the many methods which have been advocated and employed for performing tuberculin tests in man, those techniques which depend upon the development of a skin reaction are in more general use. Here, the Old Tuberculin (O.T.) of Koch was the preparation most frequently used though at present it is being replaced by a purified product.

SKIN TESTS

● The most delicate and regarded by many as the most satisfactory of these skin tests is the Intracutaneous Test of Mantoux and for routine purposes 0.1 cc. of a 1:1000 dilution of tuberculin is employed in this technique. In the Cutaneous Technique of Von Pirquet, scarifications are made, a drop of a 25 per cent dilution of O.T. is allowed to dry on these superficial abrasion and the reactions of redness, edema and other inflammatory phenomena are compared with a control. Von Pirquet's Test as a diagnostic aid is of greater value when used in children than in adults as 70 per cent of adults reveal a positive reaction. The Subcutaneous Tuberculin Test is the oldest of the methods employed in conducting tuberculin tests, having been originally introduced by Koch in 1891. It consists in the subcutaneous injection of diluted O.T. in a small dose (0.1 or 0.2 mgm.), repeating this same dose again if negative and observing local and systemic reactions. Owing to the severe reactions frequently obtained, it is considered better practice to favor and employ the intracutaneous method. In the Percutaneous Test of Moro, a small amount of an ointment (consisting of equal parts of lanolin and tuberculin (O.T.)) is rubbed into the skin on the chest without scarification. A positive reaction is shown by the development of reddening and papules as compared with the negative findings of the control. The Ophthalmotuberculin Reaction of Calmette, in which a 2 per cent dilution of tuberculin is instilled into the lower conjunctival sac is rarely used in human beings owing to the occurrence of serious accidents. In cattle, tuberculin tests as diagnostic aids are made by the intradermal ("skin") and subcutaneous ("thermal") methods and by employing the Ophthalmic or "Eye" test of Calmette.

It is to be noted that marketable *Tuberculins* are designated as *Human Type* or *Bovine Type*. The generally accepted opinion is that the bacterial protein responsible for eliciting the allergic reaction is a group antigen and is the same for the bovine as for the human type of the tubercle

bacillus. There is no evidence that tuberculins prepared from the two types differ as to their active principle and in practice there is no attempt made to differentiate specifically between infections with the human and bovine types. In most clinics the Human type of O.T. is generally employed for diagnostic testing in humans. For veterinary purposes, the Bovine type of the tubercle bacillus is employed in making such Tuberculins.

Tuberculin syringes should be thoroughly cleaned before use by boiling in soap solution and immersing for as long as possible in a sulfuric acid-dichromate solution. This is of especial importance if such syringes are also used for Schick testing or for similar purposes where skin reactions are to be read. If possible, separate syringes should be used for tuberculin tests.

DIAGNOSTIC AIDS FOR TUBERCULOSIS

● There are various stages in this disease and many types of cases in which difficulties are presented as to make it impossible to render an accurate diagnosis from a physical examination or even by other means. The use of tuberculin has, therefore, been utilized as an aid in diagnosis of obscure forms of tuberculosis, the test being applied in a number of ways. In order of their importance, the following are commonly employed:

1. Intracutaneous test of Mantoux or Mendel;
2. Von Pirquet method (cutaneous test);
3. Moro reaction (percutaneous test);
4. Subcutaneous test; and
5. Calmette's ophthalmic reaction.

Mantoux Test-an Intracutaneous Test—(*Munch. med. Wchnschr.* 1908, No. 40.) This test is employed as an aid in the diagnosis of tuberculosis. Though more difficult to perform than the Von Pirquet test, it is more delicate and the most satisfactory tuberculin test to be used in humans (*Medical Res. Council,* 1932 spec. Rep. Serv. No. 164, London).

The test is performed intradermally (between the layers of and not under the skin). Use the flexor surface of the forearm or the thigh just above the knee and slightly mesial. Controls are always carried along by injecting a sterile 50 per cent glycerin or saline solution. The total volume of liquid injected in a test is 0.1 cc., which contains as the first injection a very dilute solution of the tuberculin (1/1000 mgm.). If no reaction appears within forty-eight hours another injection is given, increasing the dose of tuberculin. The second injection is generally 0.1 cc. containing 1/100 mgm. tuberculin and the third injection contains 1/10 mgm. In case of only slight reactions, the test is repeated employing the previous and not an increased dosage. In this manner the test establishes a quantitative relationship of the degree of hypersensitiveness of the particular patient. The National Tuberculosis Association recommends when using Koch's O.T. that 0.1 cc. volume should contain the desired dose. The first injection should be 0.01 mgm. O.T. and if no reaction occurs, 1.0 mgm. is ordinarily given. In cases where extra caution seems advisable, 0.1 mgm. is used as the second injection, followed by 1.0 mgm. if the latter is negative. It is assumed that failure to react to 1.0 mgm. O.T. is sufficient evidence that there is no active tuberculosis.

The intracutaneous test is not performed upon those having fever and one-half the usual dose is administered cautiously in the feeble and in

young children. Mendel (*Med. Klin.*, 1908, *IV*, 402) originally introduced the intracutaneous test later developed by Mantoux and this test is accordingly designated by some as the *Mendel Intracutaneous Test.* Concentrated tuberculins keep well but dilutions lose strength rapidly. The latter should preferably be prepared freshly and within a short period of time before they are used. Parsons (*J. Iowa M. Soc. 28,* 69 (1938)) persents an excellent discussion of the technique of this method.

A diluent buffered to pH 7.2 with borax and boric acid, containing 0.04% gum arabic and 0.5% phenol (*J. Immunol. 34,* 103 (1938)) was found to stabilize tuberculins diluted for the Mantoux test. **Ordinarily dilutions of the tuberculins are made previous to or on the same day when the test is** performed. The same workers (*Proc. Soc. Exptl. Biol. Med., 39,* 71 (1938)) have also introduced a method for determining the potency of diluted tuberculins.

Jensen and Associates (*Tubercle,* June and July, 1938) also present an extensive study of the preparation and standardization of stable dilutions of Old Tuberculin and Purified Protein Derivative. Long and Seibert (*Am. Rev. Tuberc., 35,* 281 (1937)) recommend keeping standard dilutions of P. P. D. in the refrigerator for a period not over 3 days as they claim all dilutions lose strength upon standing. Other authorities still insist upon the use of fresh dilutions of tuberculin.

Von Pirquet Test (A Cutaneous Test) (*Wien. klin. Wochnschr.*, 1907, *XX*:1123). This was one of the most widely used of the tuberculin tests employed as an aid in the diagnosis of tuberculosis, but it is gradually being replaced by the Mantoux test, which is more satisfactory. One drop of undiluted and 1 drop of 25 per cent Old Tuberculin are placed on the sterilized skin of the front of the forearm, about 3 or 4 inches apart. Midway between the two (a control test) there is placed a small drop of sterile 50 percent. glycerin solution. The chosen areas of the arm are acepticized before any fluid is applied by cleansing with soap and water and then rubbing with alcohol and ether. The skin is then slightly scarified through each of the drops (merely removing the epidermis without drawing blood). In about five or ten minutes the excess of tuberculin is wiped away. No dressing need be applied. The areas are examined after twenty-four and forty-eight hours or if convenient every twelve hours, until forty-eight hours have elapsed. The reaction is considered positive if within these periods the tuberculin scarification shows an inflammatory area measuring at least ¼ inch, and one which contrasts markedly with the small red spot left by the control scarification. This test seems to have its greatest diagnostic value in young children. In adults negative results are of value in deciding against the existence of tuberculosis; positive results, are, however, misleading, since at times apparently healthy individuals will respond and give positive results, which may, however, be due to the fact that healed-in tubercular lesions are present.

When reading the tuberculin test in intracutaneous and cutaneous reactions, the arm should be in a good light and flexed a little at the elbow. It is advisable to look across the arm rather than down on it. When in doubt, pass the finger over the tested area as the induration caused by the edema can sometimes be felt when it does not produce an elevation that can be seen.

Moro Reaction (Percutaneous Test)— *Munchen med. Wochnschr.* 1908, *55*:216 —This test consists in rubbing gently into the clean skin of the abdomen

(some use the chest or other hairless part of the body) over an area of about 3 or 4 inches, a 50 per cent ointment of Old Tuberculin in anhydrous lanolin. A portion about the size of a pea (or about 0.5 Gm.) is employed for about one-half minute and this is allowed to remain so as to be absorbed. A positive reaction will be observed within twenty-four to forty-eight hours by the appearance of red papules. The Moro test is easy to perform and is said to be harmless. It is claimed that this test does not produce a positive reaction so frequently as does the Von Pirquet test among adults, clinically free from tuberculosis. On the other hand, a negative Moro reaction is not conclusive as is the Von Pirquet test, that a tuberculous condition does not exist. Wright and Associates (*Can. Med. Assoc. J.*, *39*, 123 (1938)) use a tuberculin ointment prepared by absorbing 1 cc. of O. T. with 1 Gm. fuller's earth and 2.225 Gm. lanolin. They recommend a more intensive application of the percutaneous test.

Many other reports have been presented concerning the use of a tuberculin ointment as a patch test. Wolff and Associates (*Am. J. Dis. Child.*, 1934, *47*, 764) consider a patch test with an ointment consisting of a highly concentrated tuberculin and killed tubercle bacilli in glycerin or an ointment base. A 96% agreement was found with the intracutaneous test and there were less non-specific cutaneous irritation and fewer untoward reactions. Also see below.

Subcutaneous Test—This test (*Munchen med. Wochnschr.*, 1930, *77*:1662; 1931, *78*: 261) depends upon the hypodermic injection of successive small doses of Koch's Old Tuberculin several days apart. The subcutaneous test is not employed to any extent in human practice today, inasmuch as there is some danger of lighting up a latent process, in addition to the possibility of disseminating the infective material. It has been replaced by safer tests.

Calmette's Ophthalmic Reaction—One or 2 drops of a 1 per cent purified Old Tuberculin is placed in the eye. A characteristic reddening of the conjunctiva will be produced within twelve to twenty-four hours if tuberculosis exists anywhere in the body. A drop of sterile water or 50 per cent glycerin solution is placed in the other eye as a control test by which to compare and judge the changes in the tested eye. This method should never be employed in the presence of any form of ocular disease. It is rarely used in human cases, inasmuch as there is some risk of injury to the eye if a severe reaction develops, though Marquez and Associates (*Soriano Klin. Mbl. Augenheilk*, 1934, 92) recommend the ophthalmo-reaction and claim it is not dangerous when carefully performed.

INTERPRETATION OF TUBERCULIN REACTION

● A positive tuberculin reaction is indicative of a tuberculous focus but not necessarily of tuberculous disease. Other methods are to be employed to find out in which part of the body the focus of infection lies, whether it is active and the extent of such activity. Failure to obtain a positive reaction, however, does not always exclude tuberculosis. Sensitiveness to tuberculin may be absent in acute miliary or generalized tuberculosis and during some acute infectious diseases. It is found occasionally that a child once a reactor may become negative to later tests. Rarely, a calcified pulmonary nodule may be seen in a child that fails to react to tuberculin. In such a case, it is probable that the focus of disease is obsolete and that no living tubercle bacilli are present.

TUBERCULIN FOR VETERINARY USE

● The tuberculin tests utilized in veterinary medicine are, in order of their importance: Intradermal, subcutaneous and ophthalmic tests. Here combination tests have also come into general use. Though the intradermal is the best and the ophthalmic reaction is the least reliable, there are times that when both are used in combination more definite and more valuable observations are available (*U. S. Dept. Agr.*, 1928. Circ. No. 249).

Intradermal and subcutaneous tuberculin for veterinary use is the same as Koch's Old Tuberculin (usually bovine strain), the former being weaker than the latter.

An alcoholic precipitate of Koch's Old Tuberculin, washed, dried, ground, mixed with sugar of milk, boric acid or bicarbonate of soda is pressed into disks and sold as ophthalmic-tuberculin disks, which are being used to replace ophthalmic liquid when performing the ophthalmic test in animals. The ophthalmic liquid is an alcoholic precipitate of Koch's Old Tuberculin dissolved in salt solution or water containing glycerin. The Bureau of Animal Industry has established definite requirements for tuberculins for veterinary use which are manufactured for interstate sale.

Tuberculin never does a healthy cow the slightest harm. It does not even hurt a tuberculous animal, but merely shows that the latter has the disease and should be slaughtered in the interest of public health. A small amount of the tuberculin is injected into the thin skin on the under side of the tail. This is examined in forty-eight and seventy-two hours. A positive reaction is revealed by the appearance of a lump.

PATCH TEST

● The tuberculin ointment patch test has been recommended by many workers. Wolff and Hurwitz (*J.A.M.A.*, *109*, 2042 (1937)) conclude that the patch test may be safely substituted for the Mantoux Test. Vollmer (*Am. J. Dis. Child.*, *56* (1938)) and Steward (*J. Pediat.*, *13*, 510 (1938)) give the same conclusions and further state that severe local and constitutional reactions do not occur and that there is no danger of infection. Hart (*Lancet*, *235*, 609 (1938)) discusses the Lederle and Copenhagen patch tests and states that though they agree in all instances when positive with the Mantoux test, they have given fewer positive reactions than the latter. Hiro (*Monatsschr. Kinderheilk.*, April, 1938) uses a tuberculin paper test, and claims that the ease in preparation of the paper and performance of the test as well as results obtained warrant its wide use. Court (*Brit. Med. J.*, *1*, 824 (1939)) in a comparative study states that the Vollmer patch test approaches in reliability the intracutaneous Mantoux test.

In performing the test, controls are used. The control material is prepared in the same manner as the test ointment except that the flasks of glycerin broth are not inoculated with the culture of tubercle bacilli. The area of application is preferably in the inner surface of the upper arm or the paravertebral region between the eighth and eleventh thoracic vertebrae. The skin at two distant points of the area is cleaned well, then washed with benzine or ether and allowed to dry.

A pea-sized drop of the test ointment is placed on one area and the control on another area. Both are covered tightly with a 1½ inch square of plain adhesive plaster. The latter is removed at the end of 24 hours. Readings are made and reread at intervals of 24 hours for a few days. The peak of a reaction is reached after 72 hours, but begins to fade after 96 hours. A weak

positive reaction will reveal a few discreet papules of a pale rose color. In a medium reaction, red papules are present and the surrounding skin is markedly red. This is also apparent in a marked positive reaction but there also will be present induration and a yellowish discoloration of the entire area. Scaling is definite up to about 10 days in medium reactions and may be seen together with brownish pigmentation of the skin for several weeks after a marked positive reaction. Itching and even a slight pain may result at the positive reaction site.

OTHER TUBERCULINS AND PREPARATIONS OF TUBERCLE BACILLI

● Originally Koch considered the soluble toxic substances of the tubercle bacillus as the desirable immunizing agents. He and others were however soon led to prepare different products from the tubercle bacillus so as to afford the possibility of producing antibacterial substances as well and as varied a supply of antibodies as possible. This led to the introduction of a large number of preparations, each product having its own advocates who claim for it superior advantages over the others. All extracts and suspensions consisting either (1) of the soluble products of the tubercle bacilli in the medium in which they are grown, or (2) containing essentially an emulsion of the insoluble fragments of the bacilli, or (3) possessing a combination of the first two are designated as "*Tuberculins*". Only those best known or most widely used will be briefly considered.

The so-called "new tuberculins" of Koch (B.E., T.R., T.O. and T.A.) are used but infrequently today.

T. R. NEW TUBERCULIN

● *Tuberculin Residue, Tuberculin Ruckst* or, *Tuberkelbacillin Rest* is made from living tubercle bacilli, dried in vacuo, and then ground to complete disintegration for many months in a ball mill (*Deutsch. med. Wochnschr., xxiii,* 209). Sterile normal saline or water is added in the proportion of 100 cc. to each gram of dried organisms and after centrifugalization the supernatant fluid, containing the soluble material, is removed. This soluble clear supernatant liquid was at one time marketed as *Tuberculin Oberes (T.O.) (do not confuse with O.T.).* The sediment or insoluble material is then dried, powdered, reground several times with water and centrifugalized after each grinding. The fluids resulting from all of the centrifugalizations, except the first, are mixed and the total volume should not exceed 100 cc. To this uncolored, opalescent liquid, 20% glycerin is added as a preservative and the resulting product known as T.R. will be found to contain 2 Mgm. of solids, equivalent to 10 Mgm. of dried tubercle bacilli in each cc. of suspension.

New Tuberculin, B.E.; Tuberculinum Novum, B.E.; Bazillenemulsion; Bacilli Emulsion. This is practically a bacterial vaccine. The culture is grown as for O.T. The bacilli are filtered out, ground but not washed, and one part of the pulverized tubercle bacilli are suspended in 100 parts of water and an equal portion (100 parts) of glycerin. It is a white, fairly permanent emulsion each cc. of which contains 5 Mgm. of dried tubercle bacilli. It is assumed that the entire contents of the organisms as well as much of the extractives are contained in this emulsion.

Tuberculin Denys; Tuberculinum Denys; Tuberculine Bouillon Filtre; Bouillon Filtrate Tuberculin. B.F. is simply a glycerin-broth culture of

the tubercle bacillus passed through a Berkefeld filter. Phenol (0.25% concentration) is added as a preservative. It is prepared like O.T. without the prolonged heating and concentration.

Tuberculoplasmin (Buchner and Hahn) is made by crushing tubercle bacilli by subjecting them to a pressure of 400 atmospheres. A cell juice in the form of an amber fluid is the result which is said to possess qualities similar to those of T.R.

Beraneck's Tuberculin, Albumose-free Old Tuberculin (A.F.), and Spengler's Perlsucht Tuberculin (P.T.O.) prepared from bovine tubercle bacilli obtained from cattle suffering with pearl disease are other tuberculins employed by workers.

PURIFIED PROTEIN DERIVATIVE (P.P.D.)

● Improvement in the quality and unifomity of tuberculin is increasing due to the biochemical investigations of Seibert (Florence B.) and others. Protein metabolic products and protein derivatives from the tubercle bacillus and from synthetic tuberculin are being made available. ***Tuberculin P.P.D.*** (*Purified Protein Derivative*) (*Am. Rev. Tuber., 25,* 724 (1932)) is obtained from standard B.A.I. synthetic tuberculin by the method of Seibert. In this technique extraneous proteins are eliminated while the substance responsible for the specific reaction is retained. It is diluted with milk sugar and prepared in tablet form so that each tablet contains a definite weight (0.0002 to 0.05 mgm.) of protein derivative. Fresh solutions are to be preferred and are made for each testing with a sterile buffered diluent supplied in each marketable package. For further details see Am. Rev. Tuberc., *30,* 713 (1934).

It appears that P.P.D. will replace O.T. for diagnostic testing, using the initial dose of 0.00002 mg. and a second strength dose of 0.005 mg. for those who fail to react to the first dose. The League of Nations Permanent Commission on Biological Standardization (*Bull. Health Org. Leag. Nations, 7,* 683 (1938)) considered it of sufficient importance to proceed and establish a subsidiary international standard for tuberculin corresponding as closely as possible to the American P.P.D. In a final report to the National Tuberculosis Association (*Am. Rev. Tuber., 35,* 597 (1937)) interesting statistical observations are reported in a summary of results of tuberculin testing with P.P.D. on a total of more than 85,000 tests. Some workers claim that the one-test method of intradermal testing with the intermediate dilution (0.005 mg.) of P.P.D. or even the smaller standard dose (0.0002 mg) is adequate to detect cases of tuberculosis infection with a high degree of accuracy (*Diseases of the Chest, 4,* 26 (1938); *Amer. Rev. Tuberc., 35,* 281 (1937)). In order to promote uniformity in reading positive Mantoux reactions with P.P.D., Aaronson (*Am. Rev. Tuberc., 30,* 727 (1934)) suggests that:

"Positive reactions may be arbitrarily classified as one, two, three or four plus, depending upon the extent of œdema measured at its widest diameters. A reaction showing some redness and definite œdema more than 5 mm., and not exceeding 10 mm. in diameter, is recorded as a one-plus (+) reaction. A two-plus reaction (++) is an area of redness and œdema measuring from 10 to 20 mm. in diameter. A three-plus reaction (+++) is characterized by marked redness and œdema exceeding 20 mm. in diameter. A four-plus reaction (++++) consists of marked redness, œdema and an area of necrosis. A reaction with slight redness and a trace of œdema, measuring 5 mm. or less in diameter, is marked doubtful. If there is no œdema at the site of injection, even if a slight redness is present, the test is recorded as negative. In interpreting the tuberculin reaction it must be remembered that redness is of less significance than the oedema."

M. Paretzky (*Arch. Pediat.*, *45*:352, (1938)) divides negative reactors to the tuberculin test into several groups. The most desirable group are persistently negative reactors with efficient immunity. The cases in this group have been exposed repeatedly to infection without acquiring tuberculo-allergy. A second group consists of former positive reactors who have lost their tuberculo-allergy through spontaneous desensitization. These patients are endowed with immunity sufficient not only to protect against disease but also to eradicate primary infection, and generally have been exposed to potent tuberculous infection. A third group is composed of negative reactors with low immunity. The ones in this group fail to react for a comparatively short period of time but infection eventually breaks the lines of defense and clinical disease develops. Pseudo-negative reactors constitute a fourth group. Tested with routine doses they fail to react, but higher dosage usually elicits a positive response. These persons are endowed with a fairly potent immunity to tuberculosis.

Human Tubercle Bacillus Protein MA-100 —(*Proc. Soc. Exptl. Biol. Med.* *27*, 661 (1930)) is a protein fraction (or metabolic product) obtained by isoelectric precipitation of the filtrate of cultures of human tubercle bacilli on Long's synthetic medium. This is supplied in sterile vials containing a definite weight of protein MA-100 per cc. A safe and efficient dosage of the latter is 0.0005 mgm. (or 1/20 cc. of a dilution containing 0.01 mgm. of the protein per cc.)

STORAGE

● Concentrated tuberculins keep well if stored in a cool, dark place. The expiration date and storage as given for O.T. are the same for all of these preparations. Diluted tuberculins even if buffered will not keep more than a few weeks. It is therefore advisable to prepare dilutions freshly or in amounts sufficient to last but for a few days and these dilutions are to be kept in the refrigerator.

IMMUNIZATION AGAINST TUBERCULOSIS

● ***Active Immunization***—It has long been known that resistance to tuberculosis appears to be dependent upon a reaction between the tissues and the tubercle bacilli. Indications point to the fact that the organisms themselves either dead or living are to be employed as the vaccine in any attempt of actively immunizing individuals against tuberculosis. For this purpose, live virulent live attenuated and dead tubercle bacilli have been advocated. In 1908, Webb and Williams employed live virulent human-type tubercle bacilli for active immunization of children by giving them minute doses at stated intervals. The fear and danger in this technique are obvious and this procedure has not and is not likely to be used to any great extent. The use of killed bacilli dates back to the days of Robert Koch. Many investigators in days gone by have used vaccines of killed tubercle bacilli in children and this procedure appears at least theoretically sound. Trudeau reported that the resulting immunity is not as pronounced as that obtained following the injection of living attenuated bacilli. Petroff however still favors the use of dead tubercle bacilli as the method of choice for active immunization against tuberculosis, expressing a belief that attenuation of virulence of living tubercle bacilli may not be permanent and that there always exists the possibility of reversion when the organisms are present in the human body. In the case of the use of

attenuated tubercle bacilli, many methods of attenuation have been introduced. At present, the use of B.C.G. on a much larger scale than employed for any similar product has focussed attention upon this preparation, the technique of attenuating the tubercle bacilli in the latter and the general subject of active immunization against tuberculosis.

B.C.G. (Bacille Calmette-Guérin).—The preventive treatment of tuberculosis by immunization against tubercle bacilli has recently received serious attention, especially in France. Dr. N. Calmette, Guerin and Collaborators of the Pasteur Institute (*Bull. de l'Inst. Pasteur,* 1924 *XXII,* 593; 1928, *XXVI,* 889) have advocated the immunization of new-born infants, where the parents are tuberculous or where environmental conditions are such that tuberculosis is inevitable, with a culture of tubercle bacilli labelled by them B.C.G. (*Bacille Calmette-Guerin*). It is not a tuberculin. This culture (so labelled) is said by them to be an avirulent or an attenuated bovine strain of tubercle bacilli, which though the organisms have lost their virulence by transplanting more than two hundred successive generations upon a medium of 5 per cent glycerin potato saturated with beef bile, they still retain their immunizing qualities. They have advocated the administration by mouth of three doses (10 mgm. to each dose) of *Bacille Calmette-Guerin* every forty-eight hours before the new-born is ten-days old. The mucous membranes of the intestinal tract of the new-born changes after the latter is ten-days old and immunization is not so successful. Though there is considerable evidence in favor of attributing a specific protective effect by the B.C.G. vaccine (*Report of Pasteur Inst. on B.C.G.,* 1932) Petroff (*Am. Rev. Tub.,* 1929, *XIX,* 9; *XX,* 275) and others claim this vaccine is to be regarded merely as a culture in which the avirulent variant R type predominates as the result of environmental conditions, and that there is always the possibility of the appearance of the virulent strain under other conditions. If this is merely a bacterial variation its universal use may be frought with danger. However a large number of reports have accumulated on the use of B.C.G. in man and animals (*Am. Rev. Tub.,* 1933, *XXVII,* 6). Kereszturi, Park and Associates in a five years study on more than 400 babies indicate that "B.C.G. vaccination is harmless and gives a very definite resistance against tuberculosis." They also advocate parenteral administration instead of its administration orally or enterally (*N. Y. State, J. Med.,* 1933, *XXXIII,* 375).

In 1927 the Swedish National Tuberculosis Association described a comprehensive scheme of vaccination with B.C.G. among the new born infants in the north of Sweden much ravaged by tuberculosis. Prof. Carl Naeslund (*Nord. med. tidskrift,* 1935, April 20, p. 616) concluded that thus far he felt B.C.G. vaccination had proven of value in the campaign against tuberculosis and nothing has happened among 7765 newborn to convict B.C.G. of any ill-effects. Other reports are presented and claims are even made by Calmette that B.C.G. immunization renders the subjects resistant not only to virulent tubercule bacilli but to other infections foreign to tuberculosis (*Ann. Inst. Pasteur,* 1933, *50,* 109, 433). Nickman (*Arch. Ped.,* 1933, *50,* 352) in reviewing the present status of immunization against tuberculosis states that "a true evaluation of B.C.G. vaccination at present is practically impossible."

More recently, Birkhaug (*Nord. med. tidskr.,* Aug. 14, 1937) who prepares B.C.G. for the Norwegian medical profession observed that B.C.G. cannot be accepted as a virus fixe. He concludes that B.C.G. never produces progressive and fatal tuberculosis. He believes instead of anticipating that

the present strains will increase in virulence, that actually B.C.G. will ultimately lose its present ability to confer immunity to virulent tubercle bacilli. He highly recommends the intracutaneous method of administration and further suggests that the usual social preventive measures should be continued. Chaussinand (*Paris med.*, *1*, 15 (1937)) reports on the use of B.C.G. and presents evidence of the innocuousness of B.C.G.

Opie and Freund (*J. Exptl. Med.*, *66*, 761 (1937)) present an experimental study of protective inoculation with heat-killed bacilli to replace B.C.G. In a more recent study, Opie and Associates (*Am. J. Hyg.*, *29*, 155 (1939)) conclude that the intracutaneous administration of heat-killed tubercle bacilli given to individuals who do not react to tuberculin result in significant protection against tuberculosis. Kinghorn and Dworski (*N. Y. State J. Med.*, *39*, 810 (1939)) in an experimental laboratory study found that 85% of rabbits and guinea pigs were protected against tuberculosis by the use of Calmette's B.C.G. (attenuated living bovine tubercle bacilli) or by the administration of Saranac caseous vaccine (dried caseous material from the lungs of tuberculous cows, containing heat-killed virulent bovine tubercle bacilli).

Cold-blooded Types of Tubercle Bacilli —There have been many workers who attempted to show a close relationship between the tubercle bacilli of cold-blooded and those of warm-blooded animals. Friedmann (*Deutsche med. Wochnschr. II*, 25, xxvi, 464) in particular gained notoriety by his claim that he succeeded in obtaining from turtles a strain of acid-fast bacilli which could be successfully employed in actively immunizing human beings. Similarly Klimmer advocated the use of the cold-blooded types for the active immunization of cattle against tuberculosis. Many workers and Kuster (*Hand. d. path. Mikroorg.*, Kolle and Wassermann, 2nd edition, V, 767) in particular have investigated the problem thoroughly. The concensus of opinion is that it is possible but very improbable that the cold-blooded types of tubercle bacilli might stimulate the production of a few antibodies which may protect against mammalian types of tubercle bacilli to a very limited extent. Most findings are inconclusive and a considerable amount of work and carefully controlled investigations are needed before it will be possible to say that the so-called cold-blooded types of tubercle bacilli (which except for the characteristics of acid-fastness have but little in common with M. tuberculosis, hominis) are of some value in the prophylactic treatment of tuberculosis in humans.

Passive Immunization—Various immune sera have been advocated from time to time for the treatment of tuberculosis. Every conceivable method has been tried to prepare such sera. It is however most difficult to immunize the lower animals to the extent of producing immune sera containing excess quantities of antibodies which might be of some therapeutic value. *Marmorek's Serum* obtained from horses immunized with young tubercle bacilli and later with streptococci obtained from the sputum of tuberculous patients and *Maragliano's Serum* prepared by the immunization of horses with toxic products of tubercle bacilli have been used. All kinds of reports have been forthcoming concerning the value of these and other therapeutic sera in the treatment of tuberculosis. The evidence however at present is very conflicting, but most workers appear to agree in the statement that up to the present time no method of passive immunization in tuberculosis has had any degree of success.

XXII

OTHER PREPARATIONS AND PRODUCTS OF BACTERIA USED AS ANTIGENS

AGGRESSINS—These products are Berkefeld filtered exudates or tissue fluids obtained from affected areas in animals artificially infected or inoculated with virulent organisms, specific causative agents of disease. These preparations representing the products of growth of the organism in the suspectible animal and also substances evolved by bacterial disintegration, are said to be of value if used properly, for active immunization especially in diseases affecting lower animals. They are sterile (bacteria-free), contain no living organisms, and therefore cannot themselves produce the initial infection. When injected subcutaneously, anti-aggressin is produced, thereby establishing protection against the infection. Aggressins are not employed for curative treatment in infected animals but are used only to actively immunize normal animals. Aggressins have been used successfully in the preventive immunization of all lower animals against hemorrhagic septicemia and as immunizing agents against blackleg in cattle.

ANTIVIRUS—A. Besredka (*Antivirus Therapy,* 1930, Paris) of the Pasteur Institute has advocated filtered bouillon cultures of bacteria in the treatment of infections by local and intracutaneous immunization and has named these filtrates "Antivirus." He states (A. Besredka, "*Immunity in Infectious Diseases*") that "two substances are present within the bacterial cell—one a thermo-labile virus which is adherent to the body of the bacterium and is the cause of infection and the possible death of the animal; the other substance is a toxin, thermolabile, is easily detached from the body of the germ and antagonizes the other substance (the virus). For convenience this substance is designated as 'Antivirus.' Experiments prove that the vaccinating power of the filtered culture is due to the presence of the antivirus in the fluid." The filtrates are prepared by growing the organisms for several weeks in broth until growth ceases. The cultures are then passed through a Berkefeld filter. Combinations of this antivirus substance are also marketed in the United States under the trade name, *A-Vee,* and various A-Vee preparations (culture filtrates) are available. The significance of these filtrates is difficult to evaluate. Schweinburg's observations (*Z. Immun. Forsch.*, 1928, *58*:53) are of in-

terest. For further consideration see Medical Research Council, *System of Bact.*, 1931, Vol. 6, p. 60, London.

Preparation of Antivirus— For specific procedures in preparing Besredka's antivirus, the following techniques are employed:

(a) From pure cultures on agar or blood agar slants, make transplants in 1% glucose broth and incubate at 37°C. for four days. Filter through a bacteria-free filter. Reinoculate the filtrate with the same organism present on agar or blood agar slants. Incubate for four days. Reinoculate with the same organism and incubate for an additional period of four days. Finally filter through a bacteria-free filter. The filtrate is the "antivirus."

(b) In this method, the glucose broth is inoculated and incubated at 37°C. for four days. Reinoculate and incubate for four more days. A third inoculation is made and after additional four days of incubation, the culture is standardized by counting the numbers of bacteria present. Heat at 60°C. for one hour. Test for sterility. Add tricresol or a suitable preservative.

BACTERIAL ENZYMES—Dubois and Avery (*J. Exp. Med.*, *54*:51; *56*:521) isolated a bacillus from a sample of soil, which under certain conditions of cultivation produces an enzyme capable of decomposing the specific polysaccharide substance constituting the capsule of Type III pneumococcus. They state this enzyme is not a therapeutic agent *per se.* However by destroying the capsule it prepares the bacteria for phagocytosis. The use of such a bacterial product in the treatment of a bacterial infection presents an interesting new avenue of research.

COLEY'S FLUID—*Coley's Mixture; Coley's Fluid; Erysipelas and Prodigiosus Toxins; Toxicum Erysipelatis et Toxicum Bacilli Prodigiosi.*

THIS fluid is prepared by growing virulent streptococci (preferably obtained from cases of erysipelas or septicemia) in bouillon for ten days at 37°C. The original formulas called for the inoculation of the latter ten-day growth with *Bacillus prodigiosus (Serratia marcescens).* After an additional ten days' incubation the mixture is sterilized in a water-bath at 60°C. and the sterile filtered product is used as the vaccine. At present the formula most frequently employed consists first in obtaining the streptococcus growth and this is sterilized separately by heat. A suspension is made of *Bacillus prodigiosus* from agar cultures and this is heat sterilized. The two sterilized suspensions are then mixed together. Both may be filtered and the toxins only employed, but more often the unfiltered mixtures are used.

Value of Coley's Fluid— Malignant tumors, especially sarcomas, have been found to decrease in size or otherwise improve or even entirely disappear in individuals who had recovered from accidental erysipelas. This old clinical observation received confirmation in the work of Fehleisen, who inoculated living cultures of streptococci (isolated from active cases of erysipelas) into patients suffering from inoperable malignant growths (not only sarcoma, but lupus and even carcinoma). This method was therapeutically applied to the treatment of inoperable malignant tumors. Coley employed toxins of streptococci and later mixtures of the latter with a heat sterilized bouillon culture of *Bacillus prodigiosus* were tried with success. There is not a uniformity of opinion in regard to the value of Coley's fluid but most workers believe that it should be tried in cases of inoperable malig-

nant growths, especially sarcomas *(Boston Med. and Surg. Jour. 172,* 321). One-quarter-minim doses are given, increasing gradually daily, until 8 minims are given at a time. The injections are administered directly into the tumor if the latter is in an accessible region or into the buttocks if inaccessible.

DIAPLYTES *(Defatted Vaccines)*—Bacteria from which the fat was removed by extraction with acetone were said to be more effective antigens (*British J. Exp. Path.*, 1921, *II*, 175; 1923, *IV*, 146). Others have reported that these defatted vaccines or diaplytes are not superior to ordinary bacterial vaccines.

The above and other bacterial preparations (as *Glycerin Vaccines* (suspensions of dead bacteria in glycerin), *Detoxicated Vaccines* (bacterial extracts treated to eliminate toxicity), etc.) have their enthusiastic advocates. The simple saline suspensions of attenuated and killed bacteria are however most widely used and appear to be the antigens of choice for active immunity.

IMMUNOGENS—*or Ectoantigens*—These are prepared by extracting young live virulent organisms for a few minutes with physiological normal salt solution. These preparations are nothing more than aqueous washings of the bacteria, and do not contain the bacteria themselves or toxin or even the culture media originally used for growing the live organisms. They contain the ectoplasm of the bacterial cells, are said to possess strong antigenic properties, are very low in nitrogen content (almost negligible), possess very little toxicity, and are employed for the same conditions where bacterial vaccines are used, but only for specific immunization. They are not recommended for non-specific protein therapy due to the low protein content. Intramuscular or subcutaneous (the former is preferred) injections of from 0.1 cc. to 0.5 cc. followed daily by increasing doses up to 2 cc. and even 5 cc. are given. A comprehensive report on Immunogens is given by Horder and Ferry *(Brit. Med. J.*, July 31, 1926), but their findings are in conflict with that of Bigger *(J. Path. Bact.*, 1927, *XL*, 261, 271).

JOHNIN—Johnin is a sterile solution containing the bacterial products of the Johne's bacillus (*Mycobacterium paratuberculosis*) and is prepared from the latter just as is tuberculin. It is employed, usually by injection intravenously into calves and cattle as an aid in the diagnosis of Johne's disease, also known as paratuberculosis or chronic bacterial dysentery of cattle.

LIPO-VACCINES—Instead of saline solution, vegetable oils were employed as the vehicles for suspending attenuated bacteria. It was thought that this would make possible the use of larger doses thus avoiding repeated injections and the slower absorption would reduce the severe reactions that occur. Favorable (*C. R. Soc. Biol.*, 1916, *XIX*, 209) and unfavorable (*Ann. d'Ingiene*, 1927, *XXXVII*, 373) reports have been presented. Available evidence points to the fact that these so-called lipo-vaccines are less effective than salt solution suspensions as antigens for active immunity.

LUETIN—Luetin is an extract of the killed cultures of several strains of the *Treponema pallidum* (*Spirochaeta pallida*), (the organism causing syphilis), which has been found to contain no impurities or foreign toxic substances and to which 0.25 to 0.5 percent cresol is added as a preservative. Based upon observations by some workers, the *luetin test* has been used as an aid

in the diagnosis of syphilis. As prepared originally by Noguchi *(J. Exp. Med.,* 1911, XIV, 99, 557), this worker and others who have employed a preparation made along similar lines have claimed that when used in the luetin test, it is of value in the examination of tertiary and latent stages of syphilis, where the reaction is most constant, though the Wassermann blood test may be negative. It is said to be usually absent or very mild in the primary and in the untreated secondary cases. In adults with congenital syphilis the reaction is said to be more marked than in infants with congenital syphilis. Alderson *(Arch. Dermat. and Syph.,* 1922, V, 610) claims that the available marketable luetin is inert and practically valueless for diagnostic purposes.

The *luetin test* or *reaction* is made as follows: After selecting a suitable area on the arm, the skin is cleansed and sterilized and 0.07 cc. of luetin diluted with an equal quantity of saline solution is injected intradermally (between the layers and not under the skin). A reaction occurs with the formation of papules, which may become pustules. A control is also performed. Barker *(Arch. Dermat. and Syph.,* 1934, III, 676) concludes that the luetin test is not reliable; and as a diagnostic and prognostic method for syphilis it is inferior to the Wassermann Test.

MALLEIN—Mallein is a preparation similar to tuberculin (which see). It is a glycerin extract containing the toxic principles of *Pfeifferella* (Bacillus) *mallei,* the causative agent of glanders. This preparation is used solely as a diagnostic agent in veterinary medicine, especially as an aid in the diagnosis of glanders in horses. The methods commonly employed to perform the *mallein test or reaction* are (1) The intradermal method; (2) the subcutaneous injection and (3) instillation into the eye (ophthalmic test). The latter is preferred in veterinary work *(Amer. Vet. Rev.* XLIV, 218). This preparation may be used for the diagnosis of glanders in humans, where the dosage is the same as employed when the tuberculins (which see) are used.

Pfeifferella (Bacillus) *mallei* is grown in glycerin bouillon at 37°C. for from two to five months. These cultures are placed in the Arnold steam sterilizer for one hour. They are then allowed to stand in the refrigerator for from two to seven days to permit settling of most of the bacteria. The supernatant fluid is now drawn off, filtered through a Berkefeld filter and the filtrate is evaporated on a water-bath to one-third of its original volume. The evaporated volume is made up with sterile 10 percent glycerin to which is added 1 percent phenol. The dose used is generally about 1 cc.

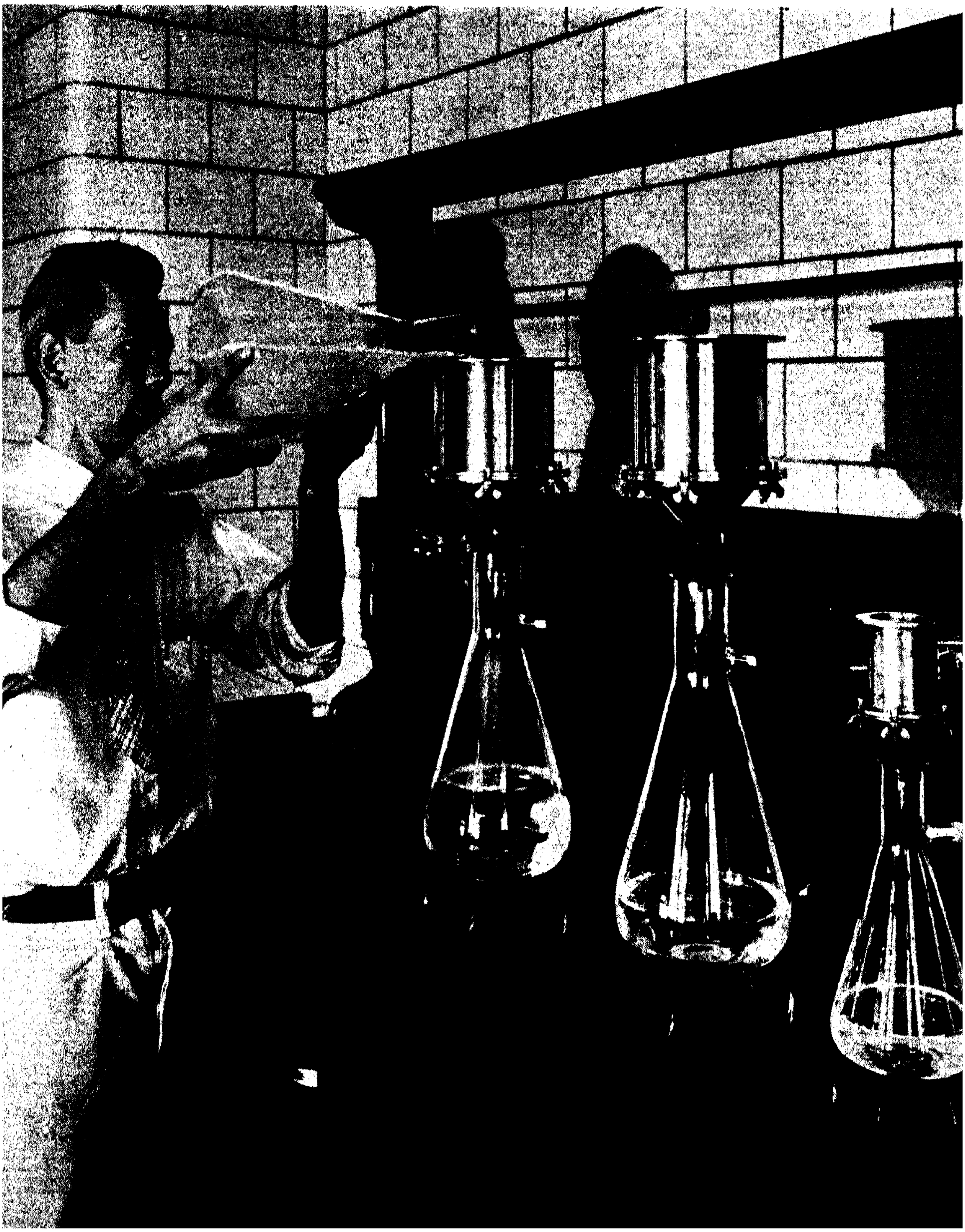
MYCOLYSATES—Gratia (*C. R. Soc. Biol.,* 1930, *104*:1058) has used suspensions of bacteria, the latter dissolved by means of a ferment secreted by certain molds or streptothrices. These so-called mycolysates have proven of value in his hands.

PHYLACOGENS—Filtrates similar to the bacterial filtrates previously considered are also marketed under the name of Phylacogens. The latter as marketed are prepared from two or more species of bacteria, employing for the preparation of the filtrate those organisms which are sup-

Grinding calf lymph preparatory to making a uniform suspension of smallpox vaccine.



Ultra filtration process which prevents the transmission of intact bacteria, but permits passage of unchanged bacterial protein.



posed to represent the species commonly found in the pathological condition for the treatment of which the particular Phylacogen is recommended. They are administered subcutaneously (occasionally intravenously) in daily doses starting with 1 cc. (or 0.1 to 0.2 cc. intravenously) and increasing each day by 1 cc. up to 8 or 10 cc. thereafter depending upon the reactions which develop and continuing until symptoms disappear. The use of Phylacogens is limited. They are said to stimulate the immunity-producing forces of the body. Phylacogens are marketed only in 10 cc. vials.

UNDENATURED BACTERIAL ANTIGENS (UBA) are suspensions or solutions of the cellular components of living bacteria which have been released by fragmentation of the latter. The technique of preparation was devised by Kruger, (*J. Infect. Dis.*, *53*, 237, (1933); (*Proc. Soc. Exp. Biol. and Med.*, *30*, 1097 (1933); *J. Lab. and Clin. Med.*, *21*, 301 (1935)). Cultures of bacteria are grown on appropriate media and the bacteria are suspended in buffered isotonic solution and thoroughly washed free of culture medium and metabolites. The final suspension is in Locke-Ringer's solution and the bacteria are counted. The suspension is placed in a special type of glazed ball mill and spun over a period of 10 to 18 hours. Residual live cells are removed by ultrafiltration through special acetic collodion membranes. Denaturation of the bacterial protein during the grinding process and ultrafiltration are avoided by special precautionary measures. The ultrafiltrate is standardized by micro-Kjeldahl nitrogen determinations.

Undenatured bacterial antigens are non-toxic. They have the same clinical application as bacterial vaccines and are said to be more specific in their mode of action than the latter. Systemic reactions are not recorded and only slight local reactions occur. Administration is by the parenteral route (subcutaneously and intracutaneously). A *Respiratory Undernatured Bacterial Antigen* (prepared from pneumococci, streptococci, staphylococci, *Micrococcus catarrhalis* and the influenza bacillus) has also been adapted to topical administration. The dosage, number of injections, and frequency of the latter vary with the different preparations and the condition treated.

Favorable results have been reported with the use of these preparations (*Laryngoscope*, *45*, 554, 856, (1935); *46*, 26 (1936)); (*J. Pediat.* *4*, 184 (1934); *5*, 590, (1934)).

XXIII

HETEROPHILE OR HETEROGENETIC ANTIGENS AND ANTIBODIES

DURING the last three or four decades there have appeared in the literature observations which were an apparent confusion to the conception or doctrine of the strict specificity of antigenic substances. For instance, it was reported that injections of sheep's blood frequently produced an immunized serum which displayed more hemolytic activity for cattle than for sheep red blood cells *(Munch. Med. Woch., 54,* 1373 (1907)). Acetone extracts of egg yolks yielded upon injection hemolysins for dog red blood cells (*Comp. rend. soc. biol. 62,* 153, 1907)). Cultures of Brewer's yeast injected into goats and rabbits produced agglutinins for strains of the dysentery group of bacteria *(J. Exp. Med., 10,* 529, (1908)). Forssman *(Biochem. Ztschr. 37,* 78 (1911)) noted that guinea-pig tissues when injected into the rabbit produced a hemolytic antibody for sheep red blood cells. It was this worker who continued these observations with extracts of different animal organs. He definitely established the fact that there existed in the organs of different animals antigens which did not follow the usual laws of species specificity. Such antigens displaying the lack of biologic relationship between the species containing the antigens and that for which the immune body displayed an affinity are spoken of as *"heterophile"* or *"heterogenetic"* or *Forssman's antigens.* The distribution of these antigens is widespread in the animal kingdom. The reader is referred for more complete details to Forssman's review on the subject (*Handbuch der Pathogen Mikroorganismen,* 1930, *3,* 470).

Heterophile antigens are found in the organs and tissues but not in the blood of guinea-pigs, mice, pigeons and horses. They are present in the red blood cells but not in the organs of sheep and of goats. Most frequently but not in all instances if present in the organs of an animal, they are not found in the blood cells of such animal and vice versa. These antigens are not found in the blood or tissues of cattle, pigs, rabbits, rats and other animals (see Forssman's review). The terms "guinea-pig type" and "rabbit type" are used at times. The former refers to animals whose organs contain heterophile antigen. Animals whose organs do not contain such antigen are at times designated as the "rabbit type."

The criterion of a heterophile antigen is its capability to cause the pro-

duction of antibodies when injected into rabbits (or other animals mentioned above), the sera of which will be hemolytic for sheep and goat red blood cells, and the responsible antibodies in turn will be absorbed by these blood cells. Heterophile hemolysins are distinct from species specific hemolysins (antibodies hemolyzing red blood cells). This can be demonstrated by the following observation which takes into consideration the fact that heterophile antigens differ from most commonly observed antigenic substances in that the former are heat stable, boiling even for hours having apparently no effect in most cases. If for instance, non-heated sheep red blood cells are injected into rabbits, both types of hemolysins are produced. If boiled sheep red blood cells are used for rabbit immunization, only the heterophile antibody is formed, and this can be removed by treatment with boiled sheep red cells or heterophile antigen from other tissue extracts.

Animals whose tissues contain heterophile antigen are biologically incapable of producing heterophile antibody. The horse falls in this class in that this species of animal does not react to heterophile antigen, but what may be of some significance is the fact that horse serum containing minute amounts of heterophile antigen may be "antagonistic", in that the latter will absorb natural heterophile antibody in man when horse serum preparations are injected as therapeutic agents. Man's tissues do not appear to possess the heterophile antigen, though Schiff and Adelsberger (*Z. Immunitatsf; 40,* 335 (1924)) claim it is present in the red blood cells of types A and AB.

HETEROPHILE PHENOMENA IN MICROORGANISMS

● The presence of substances in microorganisms which produce in unrelated species identical serological observations as with the Forssman antigen and antibody are of frequent occurrence. Reports have been presented on cross reactions between *Klebsiella pneumoniae* and Pneumococcus type II (*J. Exp. Med., 42,* 709 (1925)), and between Proteus OX 19 and the Rickettsia *(J. Exp. Med., 60,* 119 (1934)). A substance similar to the heterophile antigen has been found in various types of pneumococci, *Neisseria catarrhalis,* in certain strains of streptococci and *H. influenzae* (S type). Both smooth and rough forms of some bacteria contain the heterophile antigen, indicating that it is part of and closely associated with the somatic part of the cell. Briefly expressed it appears that the whole cell whether of animals, bacteria or plants contain a number of substances capable of acting as antigens, these differing in their chemical composition and in their immunological characteristics. The heterophile antigen for instance is regarded as a nucleoprotein-carbohydrate-lipoid complex and its immunological observations were considered above. For a further consideration of the heterophile phenomena, see *Arch. Path., 19,* 841 (1935) and *J. Immunol., 12,* 1, (1926).

MARKETABLE PRODUCTS CONTAINING HETEROPHILE ANTIGEN AND ANTIBODY

● Considerable information is available concerning the antibody response of virulent pneumococci when injected into different animals. At least four antibodies are recognized, corresponding to the type-specific carbohydrate, the species-specific "C" carbohydrate, the so-called nucleoprotein, and the heterophile antigen. The heterophile antigen is present as mentioned previously not only in pneumococci but in some strains of streptococci and *H. influenzae.* Workers, therefore, attempted the use of a

broad active immunization against heterophile antigen using this procedure against infections where the specific named bacteria are apt to be the etiological factors, as in common colds. Bailey and Shorb consider this phase in a series of several papers (*Am. J. Hyg., 13,* 831 (1931); *17,* 329, 358 (1933)) and Powell (*Am. J. Hyg., 5,* 228 (1925)) reported on the oral administration and immunization with heterophile antigen.

"Entoral," an oral cold vaccine, in which each capsule contains 25 billion pneumococci, 15 billion streptococci, and 5 billion each of *H. influenzae* and streptococci is a preparation tested before it is marketed for its high heterophile antigen content. Each of the above capsules contains antigen possessing between 80 and 100 heterophile antigenic units, which are based on antibody response as determined by adsorption tests. A *heterophile unit* as described by Bailey and Shorb is the amount of antibody which with 0.1 cc. of complement (fresh guinea-pig serum) diluted 1:4 will hemolyze after incubation 0.1 cc. of washed sheep red blood cells diluted 1 plus 3 (in terms of whole blood concentration). The relation of heterophile immunity to the incidence of colds and the use of an oral vaccine containing heterophile antigen are considered by Rockwell and associates (*J. Immunol., 28,* 475, 485 (1935); (*J. Lab. & Clin. Med., 22,* 912 (1937)).

Antipneumococcic serum containing the neutralizing antibody, the animal protective antibody and also a heterophile antibody is available as a marketed preparation. In standardizing this preparation the potency is expressed in terms of the animal protective antibody unit as described previously and formulated by the National Institute of Health. The heterophile antibody content can be determined as given above. The neutralizing antibody unit (or that amount which will neutralize 1,000 skin test doses of toxic filtrate), likewise can be determined. The heterophile antibody and the neutralizing antibody are not type specific and both are suitable for pneumococcus pneumonias regardless of type. For a consideration of heterophile antigen and antibody in antipneumococcus serum, see *Am. J. Hyg. 17,* 102 (1933); *J. Clin. Investig. 14,* 683 (1935); *Ann. Int. Med., 9,* 1664, (1936); *J. Lab. & Clin. Med., 21,* 721 (1936).

XXIV

BACTERIOPHAGE AND PHAGOTHERAPY

TWORT (*Lancet, XI* (1915), 1241) and d'Herelle (*"The Bacteriophage and Its Behavior,"* 1926, Williams and Wilkins) observed that when Berkefeld filtrates of cultures of certain bacteria are added to the cultures of the same and even other bacteria, the microorganisms disappear or go into solution. The substance which produces this transmissible lysis and apparent autolysis has been termed a "bacteriophage."

Much discussion has centered around the nature of the bacteriophage of Twort-d'Herelle. The exact nature of this bacteriolytic agent is unknown. The two leading views are: (1) That it is an ultramicroscopic filterable organism, which is widely distributed and grows only in contact with and at the expense of living bacteria; (2) That the bacteriophage is an enzyme or enzyme-like substance produced by the bacteria. Evidence presented by many workers indicates that this product possessing lytic properties of its own, is a self-perpetuating substance exhibiting a stimulating effect on homologous or closely related bacterial species. Whether it is a living parasitic organism, a product of bacterial metabolism, or enzyme, will require further investigation. d'Herelle's claim that the bacteriophage is a living bacterial species is based mainly on its capacity of unlimited self-multiplication.

Bacteriophage is very active in weak dilutions. It is produced only in contact with living bacteria (active cultures), against which it is effective and not in environments containing dead organisms, being inert against the latter. It is inactivated when heated at 72° to 75°C. for thirty minutes, and by various chemicals. It passes through the compact clay and infusorial earth filters which retain bacteria. When injected into animals, a specific antibody is produced which inhibits the lytic action of the bacteriophage, a process closely paralleling the neutralization of soluble toxin by its specific antitoxin. Bronfenbrenner has presented two comprehensive surveys and investigations of his own on bacteriophage (1928—Chapter X of River's *"Filterable Viruses"* and Chapter XL in the *"Newer Knowledge of Bacteriology and Immunology"*). Krueger (*Physiol. Rev., XVI* (1936), 129) presents a detailed review of the nature of bacteriophage and its mode of action.

THERAPEUTIC USE OF BACTERIOPHAGE

THE distinctive action (*in vitro*) of bacteriophage upon certain pathogenic bacteria has resulted in attempts to employ this as a therapeutic agent with the thought that it would destroy pathogenic bacteria in the infected animal body (*Presse. Med., 39* (1931), 168). Bacillary dysentery, bubonic plague, cholera, streptococcic and staphylococcic infections have been treated with bacteriophage preparations. The majority of the favorable reports is from cases infected with *Shigella dysenteriæ* and staphylococci. Phagotherapy has been employed for the destruction of the staphylococcus in the treatment of boils, carbuncles, and other localized infections by instillation, local, and even hypodermic injections. Results thus far reported reveal that the bacteriophage in these cases is far less effective in the living tissues (*in vivo*) than in the test tube (*in vitro*). Best results are obtained when large doses and frequent reinoculations are given (Jern and Associates, 1934, *J. Lab. Clin. Med., XIX* (1257)). Similar findings are reported by Lampert and Boyce (*Amer. J. Surg., XXIX* (1935), 436), who also mention the fact that where bacteriophage is unsuccessful it does no harm. MacNeal and Associates (*Am. J. Med. Sci.*, May, 1934, and ((1936) *191,* 170) consider the clinical application of bacteriophage in affections of the blood stream and in other abnormalities.

The marketable bacteriophage preparations are either in liquid form and found in ampuls or these sterile lysates are incorporated in water-soluble jelly bases preserved with a suitable antiseptic. They are administered topically, orally, subcutaneously, and intravenously and as frequently as clinical experience in individual cases determines. Phages specific to the particular offending agent are to be used. They are non-toxic, apparently harmless to tissue cells, stimulate phagocytosis, and are antigenic.

The true relationship of this bacteriolytic agent to disease, its treatment and prevention are still as obscure as the nature of the agent itself. More recently experimentation has revealed that bacteriophage is inactive under certain colloidal conditions, and plasma apparently inhibits both lytic action and proliferation but does not destroy the bacteriophage (Calvin, M. G., *Am. J. Hyg., XV* (1932), 247; *J. Infect. Dis., LI* (1932), 527)). The latter does not possess demonstrable bactericidal action in artificial tissue cultures, and, what is even of greater significance, is the fact that researches reveal an alleged cooperation of bacteriophage with certain bacteria to produce microorganisms of an exalted activity (i.e., organisms as *E. typhosus* and other microorganisms, unable to cause hemolysis as commonly observed, become hemolytic in character).

Eaton and Bayne-Jones (*J. A. M. A.* (1934), 103) conclude after a comprehensive study of bacteriophage therapy that "the evidence for the value of lytic filtrates is for the most part contradictory. Only in the treatment of local staphylococcic infections and perhaps cystitis (due to *E. coli* and staphylococci) has evidence at all convincing been presented. The favorable results reported may have been due to the specific immunizing action of the bacterial proteins in the material used and to non-specific effects of the broth filtrates." MacNeal and Associates (*J. Infect. Dis., 63,* 25 (1938)) believe that the clinical value is due to an opsonic effect, the organisms becoming more susceptible to phagocytosis. The marketable bacteriophage cultures have been found to vary greatly in potency (Straub and Applebaum, *J. A. M. A., 100* (1933), 110).

BACTERIAL LYSATES AND LYSED BACTERIAL ANTIGEN JELS

YOUNG actively growing susceptible bacteria in broth culture are inoculated with specific bacteriophage and incubated at 37°C., until clearing of the clouded medium occurs (usually a few hours). The lysed bacterial culture is Berkefeld filtered; potency tests are made; a suitable preservative is added; and distribution is in sterile vials. The *Lysed Bacterial Antigen Jels* are prepared by incorporating the preserved or unpreserved sterile bacterial lysate in a water-soluble jelly base.

The Jels are applied in the form of wet dressings, or tampons of absorbent cotton. Sufficient quantities must be employed to obtain good contact. Previous cleansing of the infected site should be practiced and covering the treated area with oiled silk after application of the Jel is desirable. The method of administering bacterial lysates varies with the type of lesion. Gauze dressings or drains saturated with the preparation may be applied directly to the lesion. Injections from 0.3 cc. to 1 cc. are made directly into the lesion or two 1 cc. injections are given at 24 hour intervals either subcutaneously or intravenously in cases of septicemia. Instillations of 10 cc. or more diluted to 40 or 50 cc. are given in affections of the bladder, intestinal tract, etc.

Ento-Jel is a lysed bacterial antigen jel prepared from cultures of *Staphylococcus aureus,* streptococci, pneumococci and *Micrococcus catarrhalis,* supplied in nasal-tipped collapsible tubes, and the contents are sniffed well into the nasal passages twice daily in an amount sufficient to insure good contact with the tissue cells. It is also used for ocular infections by placing a few drops in the conjunctival sac several times daily and for injection into pyorrheal pockets.

Autolysates—Autolysed cultures of bacteria have been advocated but have been found to make poor antigenic preparations. Flue (*Zbl. f. Bakt.* 1929, *113*:473) employed experimentally suspensions of plague bacilli dissolved by means of bacteriophage. Similar combinations are being tried.

XXV

MODIFIED VIRUSES (VIRUS VACCINES)

THERE is a rather large group of infectious diseases of man and lower animals that are caused by agents beyond the resolution of the microscope, incapable of being cultivated on artificial media, but which with some few exceptions are capable of passing through the pores of certain types of filters which ordinarily retain the commonly observed bacteria. These peculiar incitants of diseases or ultramicroscopic pathogens are grouped for lack of a more appropriate designation as "Filtrable Viruses". They are the etiological factors of some of the most common affections found among animals and plants. (For a detailed review, see Rivers and associates: 1928, *"Filtrable Viruses"* or Vol. 7, *"Virus Diseases in a System of Bacteriology in Relation to Medicine,"* London, 1930.) Smallpox, rabies, yellow fever, measles, mumps, poliomyelitis, encephalitis lethargica, foot-and-mouth disease, herpes, hog cholera, dengue fever, and psittacosis are some of the diseases placed in this grouping.

Various biological preparations have been marketed and presented experimentally as efficient agents for use in the prophylaxis of the above named affections. Two well-known products are modified smallpox virus, so-called smallpox vaccine and modified rabies virus, so-called rabies vaccine (which follow). In the absence of any effective antiserum produced artificially, recourse has been had to the serum obtained from human beings, convalescent or recovered from diseases caused by viruses (see also Immune Human Serums) for use as therapeutic agents either as a prophylactic or in the treatment of those diseases.

VACCINUM VARIOLÆ
Smallpox Vaccine

Vac. Var.—Virus Vaccinicum, Glycerinated Vaccine Virus, Jennerian Vaccine, Antismallpox Vaccine

"SMALLPOX Vaccine consists of a glycerinated suspension of the vesicles of vaccinia or cowpox which have been obtained from healthy vaccinated animals of the bovine species. The vesicles must be removed and the vaccine must be prepared under aseptic conditions.

The vesicles must be removed from the animal at the time of maximum potency, thoroughly triturated and made into a smooth suspension with an aqueous solution of glycerin. This solution shall not be acid to bromcresol purple T.S. and not distinctly alkaline to phenol red T.S.

Description and Physical Properties

Smallpox Vaccine is a grayish, turbid suspension; it may have an odor and a trace of color due to the presence of a preservative.

Smallpox Vaccine must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The following precautions must be observed in licensed establishments:

No Vaccine shall be prepared from any animal having a communicable disease other than vaccinia. Animals used for propagating Smallpox Vaccine must have responded negatively to a tuberculin test and, prior to vaccination, must have evidenced no ill health while in quarantine for at least seven days under daily veterinary inspection. After the vaccine pulp has been removed from each animal, a necropsy shall be performed, permanent records of which shall be kept. Each lot of Smallpox Vaccine shall be examined to determine its freedom from undue bacterial content, and a special examination shall be made of each lot to determine the absence of tetanus organisms and other pathogenic anaerobes. Permanent records of these examinations must be kept. The finished product must be placed in sterile containers that comply with the requirements of the law and of the regulations established by the United States Public Health Service.

The outside label must bear the name *Smallpox Vaccine,* the name, address, and license number of the manufacturer, and the date beyond which the Vaccine may not be expected to retain the potency prescribed by governmental authority. The label must also bear directions concerning storage of the package below 5°C.

Storage

Preserve and dispense Smallpox Vaccine in hermetically-sealed, capillary glass tubes.

It must be kept at a very low temperature, preferably below 0°C., and never above 5°C. as it loses potency rapidly at higher, even moderate, temperatures.

The requirements for labeling are that the outside label must bear the name SMALLPOX VACCINE, the name, address, and license number of the manufacturer, and a statement of the time beyond which the vaccine should not be expected to retain its full activity as prescribed by governmental authority. The label must also bear directions concerning storage of the package below 5°C.

Smallpox Vaccine loses potency rapidly, even at moderate temperatures. It must therefore, be kept at the lowest possible temperature, preferably below 0°C., and never above 5°C.

SMALLPOX

● Smallpox, a highly contagious affection and one of the most fatal of the acute diseases, has been a scourge of mankind attacking all ages and all races, prevailing in all countries over the entire globe and occurring throughout all periods of man's existence. At one time it was more common than measles, was the most common cause of blindness and during the 18th century, of which the best records are available almost everyone had the disease before the age of seven or before adult age. During this period, it

is estimated that more than 600,000 died annually or a total of sixty million deaths due to smallpox were recorded during this century. Dreaded then as now, this scourge is a loathsome disease for those who recover are most frequently mutilated or disfigured, maimed or deformed. It granted nor grants favors to anyone and the unsightly permanent pitting or scarring of the face is almost always present except in the very mild cases. The nature of the agent which causes smallpox is still unknown and is spoken of as a virus. The diseases smallpox, cowpox, horsepox, and sheeppox, if not identical are closely allied. The first two smallpox or variola and cowpox or vaccinia if not one and the same condition are at least very closely related.

VARIOLATION OR INOCULATION

● Our knowledge concerning protection against smallpox is fortunately much more advanced than is that of the cause of this malady. Vaccine virus (see the U.S.P. description given above), is available today as a sure and complete safeguard against this dreaded scourge. It has been known for centuries that one attack of smallpox usually protects against subsequent attacks (though not necessarily in all cases). This knowledge was made use of in what became known as "variolation" or "inoculation" or exposing young healthy children to infection during mild outbreaks of smallpox, hoping that mild attacks of the disease would result which would confer a lasting resistance or immunity. This old custom of inoculation was practiced by the Chinese from time immemorial. In April 1721, Lady Mary Wortley Montagu returning to England from Turkey introduced to the English more generally the practice of inoculation for smallpox after her own son had been "engrafted" with a successful outcome. This practice became popular on the continent and spread to America when the Rev. Dr. Cotton Mather of Boston suggested its use to combat the ravages of smallpox. "Taking smallpox by way of inoculation" became a speciality and inoculating hospitals were opened in isolated districts. As each succeeding outbreak of smallpox served to demonstrate that those who were previously inoculated were immune and had not contracted the dreaded disease during the existing epidemic, the practice of this method of immunization progressively gained ground in spite of even bitter opposition. The technique of "inoculation" ("variola inoculata" or "inoculated smallpox") was practically identical with that of "vaccination" as used today. The difference lies in the material employed in the technique of immunization. In the inoculation procedure, the matter is obtained by dipping ivory splinters into the fluid of the vesicle or pustule of a case of active smallpox. These ivory or bone points with the dried material were rubbed into the scratched area of the skin in the individual to be inoculated. The mild disease which usually develops is however just as communicable as true smallpox and those who may contract the infection in this manner develop true smallpox which may reveal itself in a serious form and even prove fatal. ***Inoculation Results in Creating an Immunity in the Individual Thus Protecting Him But it May Endanger the Community at Large.*** While dangerous in the extreme, "variolation" or "inoculation" nevertheless was not without a considerable amount of benefit. But this practice as a universal and lasting method of choice to produce an immunity against smallpox was made unnecessary by the classical work of Edward Jenner of England published in 1798.

VACCINATION

● Jenner proved by a series of interesting and carefully conducted experiments that cowpox or vaccinia (coming from the Latin *"vacca"*,

meaning cow), a very mild disease to which many animals including man are susceptible, is readily transferred and that an attack of this disease and the immunity created protect against smallpox. The causative agent of this condition is spoken of as vaccine virus and is to be found in the material present in the skin eruption of those animals having vaccinia or cowpox. The term "vaccination" then possessing a specific meaning came into use and referred to the method of transferring the virus from the skin eruption of an animal having vaccinia or cowpox into the skin of another animal. Such vaccine virus is obtained today from calves, but may also be obtained from older cattle, goats, horses, mules, rabbits and other mammals. Dr. Benjamin Waterhouse, professor of medicine at Harvard was the first to introduce the practice of vaccination into this country (in 1800), employing vaccine virus obtained from England. In the early days the vaccine virus was obtained from cattle and from human sources, but the latter is rarely employed today due to the fact that the supply would not be sufficient and the "arm to arm" vaccinations also present the possible danger of transmitting other human diseases. In some countries however the use of the human virus in the arm to arm vaccination technique is still practiced. It was in 1860 that vaccination with the "animal lymph" of calves came into more general use, although it was employed more or less since the time of Jenner. Since 1866 when the Beaugency strain was discovered and especially after 1891 when glycerin was employed for purifying and preserving it, the "Bovine Virus" was and is employed mainly for the yield of the present-day smallpox vaccine. This makes possible a constant fresh supply and in amounts as may be required. Such a preparation was first admitted into the United States Pharmacopeia in the Ninth Revision.

SEED VIRUS

● The initial material used for preparing the calf virus is designated the "seed virus". This can be obtained from vaccination and pustules in children, from spontaneous cowpox, or from pustules in calves after several passages of smallpox virus through calves. The virulence of smallpox vaccine virus is usually maintained by intermittent passages through rabbits. The human-rabbit seed is generally used as it is efficient, reliable and easily obtained. In its preparation, crusts are collected from healthy children about nineteen days after successful vaccination. These crusts are cut and emulsified with boiled water to a mucilaginous paste. This humanized seed is inoculated into an area about six inches square upon the abdomen of a calf, the remainder of the calf being vaccinated in the usual manner. The pulp from this special area is separately collected and glycerinized as detailed below. It is then tested bacteriologically and clinically. The bovine virus from human seed is now used in a dilution of 1 part to 12½ parts of distilled water to vaccinate rabbits. The seed is rubbed upon the skin of a rabbit's back which has been freshly shaven and cleansed thoroughly with sterile water and alcohol. Five days after vaccination the pulp is removed with a curette, weighed and emulsified in a mortar with a solution consisting of glycerin 50%, sterile water 49.5% and carbolic acid 0.5% in the proportion of 1 part of pulp to 8 parts of the solution. Four rabbits should yield from 15 to 20 cc. of this emulsion—an amount sufficient to vaccinate one calf.

PREPARATION OF VACCINE VIRUS (CALF VACCINE)

● The animals in general use for the preparation of vaccine virus are young carefully selected calves preferably females (though this is immaterial) but they must be at an age of from four months to one year. The younger animals are preferred (2 to 4 months). They are kept under rigid veterinary observation and supervision for a few weeks previous to and during their actual treatment to insure freedom from disease, the tuberculin test also being carried out, and any reactors are eliminated. The calves are kept under the best sanitary conditions which can meet all known scientific exactness for the latter. This includes the greatest cleanliness in regard to the bedding and stalls. All dry fodder and bedding passes through a steam sterilizer before use. The stalls are disinfected before occupation. The stable located at a distance from other buildings on the premises is generally of stone and cement construction, floors are of cement, and walls are enamelled with the exception of those in the operating and incubation room which are tiled. Separate quarters are provided for housing calves under treatment and for animals held under observation or during the quarantine period. The stables are so built that they can be easily scoured and flushed with hot water and a comfortable temperature is maintained at all times. The calves are fed either with an exclusive whole milk diet or hay and grain when necessary. When first brought into the stable, the hair is clipped from the entire body of the calf and the latter is washed and cleaned thoroughly including the feet and tail. A day or two before vaccination, the posterior abdomen (extending from the ensiform cartilage to the pubic region including the entire width of the belly) and insides (inner folds) of the thighs are shaved or depilated. Just before the actual vaccination, the animal is strapped to a specially constructed operating table in such a manner as to allow free access to the shaved area. The latter is washed in succession with soap and water (some follow with a weak solution of lysol, then with sterilized water and alcohol), and finally dried with a sterile towel. On this area, superficial linear incisions are made, and after the latter are dried with sterile cotton or gauze or a sterile towel, there is rubbed in a previously prepared virus which is known to be active (and designated as the "seed virus"). Vaccinated calves are kept in pens constructed of cement and stainless steel and during the incubation period the vaccinated area is kept clean by spraying with sterile water or some employ daily sprays of a 1 to 500 solution of brilliant green. The latter has no appreciable effect upon the potency of the virus but acts as an efficient bacteriostatic and bactericidal agent. After the typical vaccinia vesicles develop (usually six or seven days after vaccination), the entire contents of the latter including the serous fluid or lymph and pulpy exudate (but minus the crusts which are removed) are collected under rigid aseptic conditions, employing humane operative procedure. The pulpy mass is ground thoroughly under aseptic precautions in a special mill making an even emulsion with four times of its weight of 50% glycerin containing 1% carbolic acid. This finished mixture is stored for 3 to 4 weeks at very low temperatures to permit bacteria which may be present to die. Every lot of vaccine is then subjected to rigid bacteriological and toxicological examinations to insure a preparation free from all disease-producing organisms (especially anaerobes and hemolytic streptococci) and toxic substances. Autopsies of the calves employed in preparing the marketable virus are made and no virus is used until necropsy reports reveal that the animals were in perfect health. The preparation is then tested on rabbits

and susceptible humans to prove that the virus yields a successful "take", the actual strength or potency being estimated from the speed of the development and the extent of the subsequent reactions and compared with a vaccine of known high potency inoculated on the opposite side of each rabbit. After these operations and tests are conducted exercising every possible care, the preparation is filled mechanically in vacuum jars under rigid aseptic conditions in sterile capillary tubes and then released for the market. The sterile capillary tube sufficient to hold one human dose is the only form of dispensing allowed in this country and Great Britain. A calf usually yields about 10 Gm. of pulp which when made up will produce material to vaccinate about 1500 children.

Orskov *(Bull. Health Organ., Leag. of Nat. 7,* 679 (1938)) considers the question of the standardization of smallpox vaccine.

PREPARATION OF SMALLPOX VACCINE (other than from calves)

● The present day calf smallpox vaccine production has resulted due to Jenner's original presentations. Smallpox is transmissible to cattle. The disease is altered in its passage through several animals, the virus becomes attenuated and symptoms approaching a typical cowpox result. The virus from calves thus affected gives rise in man to mild local lesions which never produce smallpox but protect against exposure to this disease. The desire, however, has been widespread to obtain a marketed vaccine lymph which would be potent and efficient, yet free from bacteria and other contaminants. Various culture vaccines have been presented. Only recently Plotz *(Compt. Rend. Soc. Biol., 130,* 152 (1939)) employed an antivariola culture vaccine to vaccinate 11 children and concluded that it confers a lasting immunity.

Goodpasture and Associates (*Science, 74,* 371 (1931); *77,* 119, (1933)); *(Am. J. Hyg., 21,* 319 (1935)) introduced the use of the chorio-allantoic membrane of the 14 day old chick embryo for the production of a vaccine virus, thus eliminating the hazard of bacterial contamination.

PRODUCTION OF SMALLPOX VACCINE IN THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO

● This product is now marketed and available for vaccination against smallpox. Potency tests give results which compare favorably to those produced by potent calf smallpox vaccine. Its antigenic quality is as efficient *(Am. J. Pub. Health, 27,* 1135, (1937)). The following details the method of production:

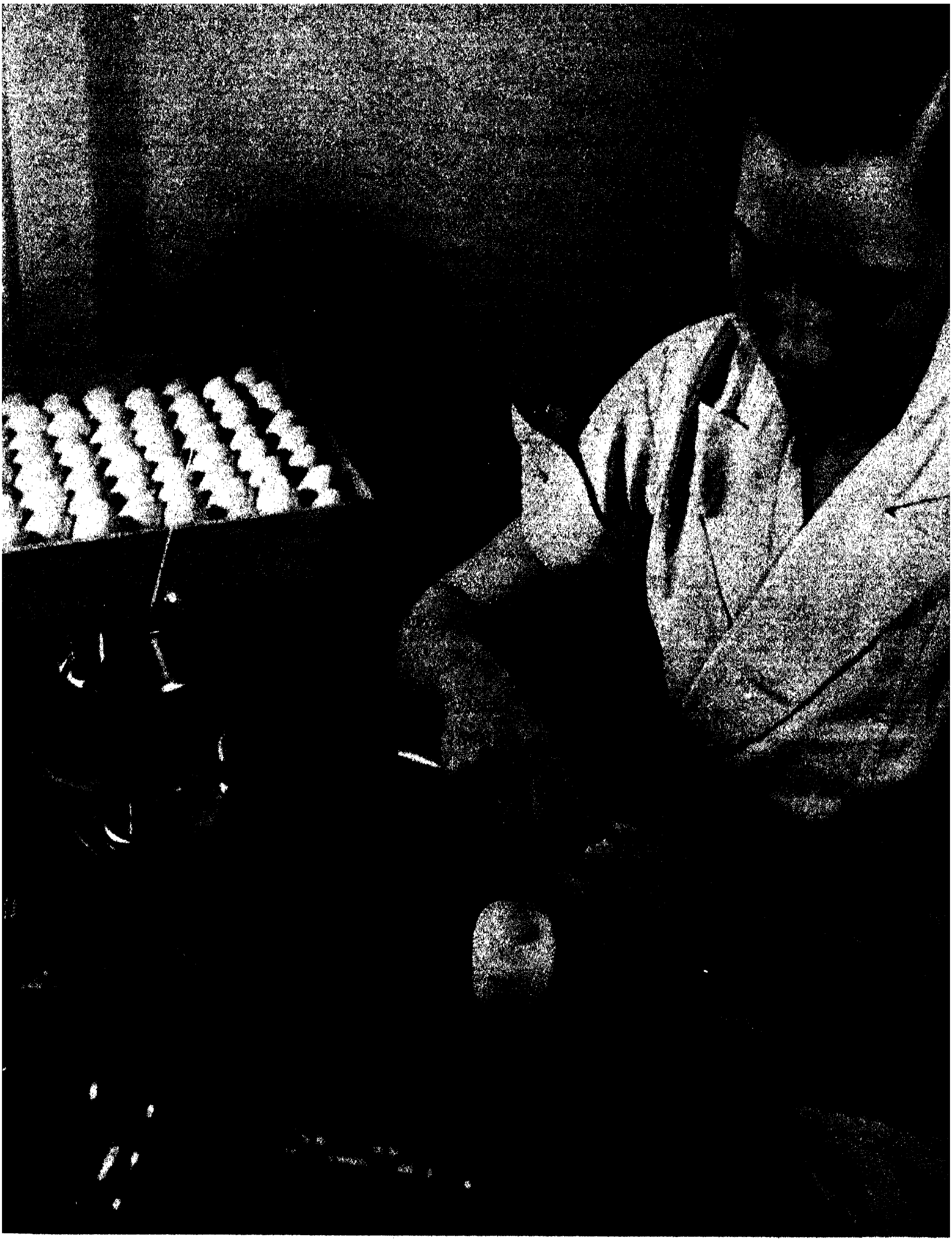
Two methods of procedure are employed: the cover-slip method used mainly in investigative work and the shell-flap method employed for large scale production of the virus. Large, well formed hen eggs carefully selected with firm shells and possessing a high percentage of fertility (obtained from a controlled source) are marked with the date. They are placed in a suitable or standard laboratory incubator at 39°C. (at the bottom of which is a shallow pan of clean, fresh water to supply the proper humidity). After 24 hours incubation, the eggs are turned and cooled at least once daily (for the first five days merely by timing them). Eggs thus treated and incubated from 12 to 15 days are most suitable for vaccinia culture, a 14 day embryo being most satisfactory. On the 13th day the eggs are candled in a dark room and all infertile eggs and dead embryos are discarded. After candling the germinating eggs are gently washed with castile soap and luke warm water (40°C.) and then placed in a separate incubator to dry and to become adjusted to the temperature. The eggs are kept in position by being placed in removable metal trays constructed to a diamond-shaped mesh of

heavy galvanized wire (1½ inches each way), under confined area and placed upon a suitable support, and the shell directly over the embryo is opened carefully without injuring the underlying membrane. Various devices are available for cutting the opening in the egg shell but a dental motor flexible shaft and carborundum disc equipment serves best for the purpose. A window about 1 inch square is cut and then covered with melted paraffin (60-62°C.). The eggs are returned to the incubator at 37°C. with the window side up. The inoculation is performed on the following day, by first applying a fresh layer of melted paraffin over the cut and area directly surrounding it. With a sterilized dissecting needle the shell membrane is cut through, the base being left intact and the chorio-allantoic membrane is thus exposed. Inoculation is made by rubbing bits of infected tissue lightly over the membrane. The shell flap is then carefully returned to its former position and sealed over with a thick layer of melted paraffin (not over 60°C.). The vaccinal lesion on the chick embryo is removed from the egg after an incubation period of 72 hours; dead embryos are discarded. Under aseptic conditions swabbings from the lesion are made for bacteriological tests and the lesions are then removed. Contaminated lesions are discarded. Sterile lesions used to prepare the vaccine are pooled and ground quickly while frozen until a homogenous pulp is obtained. Four parts of sterile 50 percent neutral glycerin in saline is added to each part of ground pulp and the finished vaccine is stored in capillary tubes in the refrigerator at 0°C. Sterility, safety and potency tests are made. A weak preservative is added by some laboratories (as merthiolate (1:10,000)). Glycerinated chick virus loses potency rapidly at temperatures higher than 5°C.

EGG-EMBRYO-TISSUE VACCINE

● Rivers and Ward (*J. Exp. Med. 58*, 635 (1933)) employed a tissue-culture method of preparation using a medium consisting of 0.1 gram of viable chick-embryo tissue suspended in 4 to 5 cc. of Tyrode's solution. Successful vaccinations were made on 100 individuals. Herzberg (*Z. Immunitatsforsch., 86*, 417 (1935)) made an elaborate study using the Tyrode-egg embryo-tissue and the egg chorio-allantoic membrane cultures and preferred the former preparation. Both of these products have been used intradermally with satisfactory immunological results. However, there is no scar left after using the intradermal technique. This may mitigate against an intradermal procedure as health workers would have to depend upon the history obtained from the patient rather than findings (as scarring) which are definite proof. The tradition of the use of calf lymph, reliable and dependable, may be difficult to uproot and more than likely the Tyrode-egg embryo-tissue and the egg chorio-allantoic membrane culture vaccines will find but little use in practice at the present time.

Rivers and Associates (*J. Exp. Med., 69*, 875 (1939)) in a recent study concerning the amount and duration of immunity induced by the intradermal inoculation of cultured vaccine virus conclude that the intradermal inoculation of a vaccine virus attenuated by cultivation on Tyrode's solution and minced chick embryo does not confer a sufficient lasting immunity for practical purposes. Until a cultured virus will be developed which will give an enduring immunity in all cases, they suggest that smallpox immunization be performed by the use of a single intradermal injection of cultured virus followed within ½ to one year by the ordinary vaccination with calf vaccine. The advantages in this procedure are that only a very small superficial scar will be produced and there will be no fever and discomfort which frequently



A step in the manufacture of chick-embryo vaccine virus. Preparing egg for inoculation.



Completing the egg-inoculation in manufacture

of chick-embryo vaccine virus.

accompany a primary ordinary vaccination. Ellis presents findings in a comparison of different vaccines and various techniques in the immunization of over 9,000 students (*Pub. Health Repts.*, *54*, 1012 (1939)). Three kinds of vaccine were used, two cultured by the methods of Rivers and of Goodpasture, and one prepared by the usual calf method. The River's vaccine was employed intracutaneously. Goodpasture's vaccine was employed both intracutaneously and by the multipuncture technique. The calf vaccine used only by the multipressure technique gave the greatest number of primary takes. However, the results with the Goodpasture chick vaccines used intracutaneously were not grossly inferior to those obtained with calf lymph. Severe local and constitutional reactions following vaccinations were slightly less frequent with the chick vaccine (Goodpasture) than with the calf lymph vaccine. Severe local and constitutional reactions were reported more frequently with River's vaccine given intracutaneously than with any other type of vaccine. There was little difference in the size of the scar produced in the primary takes with the various kinds of vaccine or methods of vaccination. There was little difference in incidence of scarring in the different methods used.

Dried Smallpox Vaccine (powdered vaccine virus in vacuum tubes) is an active and potent virus dried under vacuum at a temperature of approximately –10° C., ground and passed through a sterile sieve and finally hermetically sealed in vacuum. It is of special value in tropical and subtropical areas where the glycerinated lymph would quickly lose its potency. In using this powder a homogenous paste is made by mixing with sterile distilled water *Immediately Before Use.* Dried smallpox vaccine, preserved in a vacuum may be given an expiration date not to exceed 6 months from date of manufacture (or date of issue).

KEEPING SMALLPOX VACCINE

Smallpox vaccine, so-called vaccine virus or vaccine lymph, cannot be injured by freezing. The U. S. Public Health Service requires that the date of expiration be marked plainly on each package (marketed). This indicates the expectancy of potency (efficiency) when kept under favorable conditions. The colder the temperature, the more assured will one be of the efficiency of the product. It should be kept well below freezing if possible. Based on the governmental regulations, the expiration date usually stamped on each package of smallpox vaccine is not more than three months from the date of issue, if the product is kept below 5°C. (41°F.). If the temperature provision is not given, the expiration date is only one week from the date of manufacture (or date of issue). The date of issue (which may be more than two years after date of manufacture) can be used instead of the date of manufacture only if the product is kept constantly below 0°C. and "provided that the filled and completed product, when tested for potency within nine months of the date of issue by the technic described in Hygienic Laboratory Bulletin No. 149 (pages 12-16) meets the minimum requirements for virus stored less than two years, and provided further that it shows no material reduction of potency as compared with a lot of freshly prepared highly potent vaccine used on each rabbit for a control."

Tests have shown that a potent vaccine refrigerated constantly at 10°F. will retain its activity for four years. Kept at 50°F., (or 10°C.), the temperature of the ordinary house refrigerator, the potency remains for from three to four months, while at room temperature (70°F. or 21°C.),

(as in a drawer, case or bag), the average life of vaccine virus is from one to three weeks and the number of "takes" is reduced to 50%. If the vaccine virus should be placed in one's vest pocket or close to the body or exposed to summer heat, its complete value will be lost within three days and "takes" will not result. Kept accidentally near a stove, radiator or near steam pipes as might happen during delivery or mailing when packages are carelessly thrown about and where temperatures may approximate 120° to 140°F., (or 49°C. to 60°C.), the potency will be destroyed within 5 minutes. Once the strength or activity of vaccine virus is destroyed or weakened, it cannot be restored by any known treatment. It is therefore usually advisable to obtain from the manufacturer the smallest practicable amount which may be needed at any one time, unless below freezing temperatures for keeping the smallpox vaccine is available or possible.

USES

● Smallpox vaccine is not used in the treatment of active cases of smallpox. Its primary value is for the prevention of this dreaded disease. Quarantine, isolation and disinfection will aid in checking the spread of the latter to a certain degree but successful vaccination and revaccination will prevent individuals from contracting this scourge. It is almost impossible to approximate the exceedingly large number of lives saved and the extent of the misery avoided in the protection afforded by vaccination against smallpox with smallpox vaccine.

TECHNIQUE OF VACCINATION

● Vaccination is a simple operation, yet one which requires some skill in performance and care in after treatment. Any method may be employed providing however that the vaccine is deposited in the deeper layers of the epidermis (superficial skin) covering a small area, not larger than ⅛ inch in any direction, and avoiding the drawing of blood or any marked irritation of the skin. The method of incision or linear abrasion, the drill method and best of all the multiple pressure or multiple puncture method are employed. When using either of the first two methods, the arm after vaccination should be exposed to the air *(But Not To Direct Sunlight)* for one-quarter of an hour before the clothing is allowed to touch the dry area. In case of the multiple pressure method, the remaining glycerinated vaccine can be wiped off immediately and with this method "control" sites are not necessary as when other techniques are employed. In preparing the site for vaccination, the skin in the area to be treated is thoroughly cleansed with soap and water or with alcohol or preferably with acetone on sterile cotton or gauze and then wiped dry. In vaccinating, antiseptics or even soap are not necessary, but if used all traces of the latter are to be removed with boiling water from the area to be treated and the latter is then dried; or if a volatile antiseptic is used, such chemical agent should be allowed to evaporate. Not only heat, but antiseptics also kill or reduce the potency of vaccine virus. Injury to the vaccinated arm should be avoided. Tight sleeves or any tight covering which tend to macerate and inflame the tissues during the "take" should not be allowed. Do not use pads, plasters, adhesive or "vaccination shields" (made either of celluloid, felt or similar material) as they act as a poultice, and thus gather heat and moisture, two objectionable features. The wound should be kept clean, cool and dry. It is best to wear a loose-sleeved white garment. The best dressing is the unbroken skin and

the scab which subsequently forms. If dry sterile gauze is applied for the first few days after vaccination, this should be removed before the pustules appear.

Vaccination of infants is attended with less outward effects and possible complications than that of older children so that vaccination should be performed before the child reaches one year of age and as soon after birth as practicable, preferably before teething. Avoid vaccination in the presence of eczema and especially when acute infections of the central nervous system are particularly prevalent. Vaccinate if possible during the cool weather, the late fall, winter and early spring (October to May) being the most suitable seasons. Not only is the quality of the vaccine likely to be better during cold weather but during the latter period children are less apt to play with bare arms or legs and thus the possible risk of infecting the vaccination wound is reduced to a minimum. Vaccination on the arm is to be preferred (outer surface of the left arm at about the insertion of the left deltoid muscle is most suitable) because of the dryness of skin, drainage by lymphatics and less risk of traumatism. In leg vaccinations (especially with babies in diapers and with young children) the wounds are exposed to more moisture, to greater contamination from street dust and to possible injury and subsequent complications (frequently there is extensive ulceration). If the leg is selected, the site chosen should be on the calf below the head of the fibula and not on the outer surface of the thigh. If the vaccinated area is kept dry and cool, (keep away from heat and moisture), it is not necessary to modify the routine care and feeding of the vaccinated individual.

SUCCESSFUL VACCINATION

● Vaccinations should always be inspected to be assured of a successful "take" which in turn is an assurance that the individual is protected against smallpox. It is of interest to note that an immunity is also produced against cowpox.

Smallpox Vaccine Virus of *Full Potency* when applied (by any of the techniques) to the skin incites a localized and mild form of the disease (cowpox) from which the patient recovers rapidly. The types of reaction following vaccination and the immunity (resistance or protection) created can be judged and indicated only if vaccine virus of full potency was used. Where there is an absence of immunity to smallpox prior to vaccination, the reaction produced is spoken of as a typical *Primary Vaccination or Vaccinia.* One or more small, round, red, hard papules appear at the site of inoculation within a few days, or by the fourth day. These become vesiculated on the fifth day and are surrounded by a zone of redness (or areola) which may appear on the following or within a few days, and though rather narrow from the third to the seventh day it begins a sudden spread on the seventh day (increasing about 1 mm. in diameter daily), after vaccination and reaches its maximum (of about 12 mm. in diameter) in from 8 to 14 and usually in nine to ten days. There is generally but one vesicle, clear and pearl-like surrounded by a deep, red, and even swollen areola, a picture of the "pearl upon the rose leaf". The skin may feel hot and be painful. The auxiliary nodes or glands may become enlarged and tender. Soon after the tenth day the areola (or erythema) begins to fade, the swelling subsides, and on the following day the pustule dries rapidly, becomes brown and crusted, the wrinkled scab separating and dropping off in two to three

weeks (usually about the 20th day). Systemic symptoms and indications of a mild febrile reaction may appear but they disappear quickly. In highly susceptible patients, a rash covering a large portion of the body may develop (eruptive vaccinia), but disappears quickly.

If the patient has some degree of or partial immunity as would be the case in an individual who has partially lost the protection gained from a previous vaccination or an attack of smallpox or natural vaccinia, a modified or accelerated reaction, known as *vaccinoid,* results when vaccination is practiced. The reaction is less severe and develops more quickly, reaching the maximum in from three to eight days. A vesicle is generally found at the center of the reaction, the areola being most striking. Scars left in cases of vaccinoid are much less marked.

In highly immune individuals, as would be found in persons fully protected against smallpox by recent vaccination or a previous attack of smallpox or cowpox, the maximum diameter of the erythema is reached and passed in from 8 to 72 hours. There is usually no vesicle and scars are not usually produced. It is of interest to point out here that in various parts of the United States, mild smallpox has been prevalent among the unvaccinated and the condition was diagnosed as "chicken pox." Such individuals will give no evidence or history of previous successful vaccination or an attack of smallpox. Subsequent vaccination will yield the *immune reaction* described here.

The three types of successful vaccinations, (primary vaccinia, vaccinoid, and immune reaction), are an arbitrary differentiation based on the time when the maximum diameter of the erythema (broadest redness) is reached. It is therefore important to observe and measure (if necessary) at intervals of two days after vaccination the diameter of the redness. The cooperation of the person vaccinated may be needed to observe when the maximum reaction is obtained. There appears to be a definite relation between the immunity conferred and the diameter of the erythema when at its maximum in the area of the local eruption; and furthermore the degree and duration of the immunity are directly proportional to the typical nature of the "take" (being assurred of course that a potent vaccine virus and the proper technique were used in vaccination). The amount or degree of redness and the time of the first appearance of the latter during vaccination are not a measure of the state of immunity in the subject. The *Quicker* the maximum reaction is attained and then subsides the greater is the immunity which prevails. It is therefore the *Time* which is required for the area of redness to reach its *Maximum* which serves as the index of the existing immunity.

NON-TAKE

● It is a fallacy to state and yet such statement is frequently quoted that if a vaccination fails, the subject is immune. The eminent Sir William Osler though repeatedly vaccinated never had a successful "take". Supposedly immune, he worked hard in the smallpox wards in the Montreal General Hospital and also mingled freely with friends and relatives. It was in December 1875 he himself contracted the dreaded malady (though a mild attack) and in the years to follow he always cited his own case to illustrate the fallacy of the "non-take" belief as an evidence of immunity which prevailed at that time.

Every vaccination if performed properly (preferably multiple pressure method) and with a fresh and potent vaccine virus should give a reaction as indicated previously. If the papule and areola do not appear until the fourth

day and there is no vesiculation, either the technique was unsatisfactory or more than likely a weak vaccine virus was used. In case there is any doubt as to the potency of the virus, either use another fresh virus which has been kept cold or employ one of known potency. If this is not possible, then to guard against failures vaccination should be performed at more than one site at least an inch apart, keeping each area of the minimum size. The failure of a vaccination to "take" does not indicate a protection against smallpox. Differences in skin receptivity independent of the existence of an immunity are known to exist, especially in infants and young children. It has been observed that some very young infants are not as easily vaccinated successfully as are most of the older children. However they have been found susceptible to smallpox. In still rarer instances, some individuals have been found resistant to specific batches of vaccine virus, the latter generally yielding successful "takes" in others. In both instances vaccinations with potent lots of vaccine virus should be repeated until "takes" are successful.

RE-VACCINATION

● It is possible for some non-immunized individuals to be exposed to smallpox, especially to the milder forms, without contracting the disease. This is not however a definite evidence of the existence of an immunity. A successful vaccination especially if recent will protect almost entirely against smallpox. If the successful vaccination had been performed only once previously and a long period of time has elapsed before exposure to an outbreak of a severe epidemic, contraction of the disease, if at all, will result in a suppression of fatal and serious symptoms and smallpox only in a mild form will prevail. The possibility of contracting smallpox varies with the intensity of the exposure, with the severity of the strain of smallpox to which one is exposed, and with the susceptibility of the individual. It is impossible to attempt to fix a definite time for the duration of an immunity conferred by a successful vaccination. This varies as in other infectious and immunizing proccesses. It is a known fact that the immunity wears off after a number of years, being absolute for the first few years, beginning to disappear two years after vaccination and in most cases almost completely lost after a ten year period (average duration of immunity is regarded as seven years). In this as in other diseases immunity is a relative term.

Health officers who are continuously exposed and those living in areas where smallpox is prevalent need more frequent vaccination than those who are not exposed to this disease. For the public at large in this country, two successful vaccinations are usually advised and are regarded as being sufficient to protect for life against the milder forms of smallpox which are the types most prevalent in this country. The first successful vaccination is to be performed before the child reaches one year of age or as soon after birth as practicable, preferably before teething (at age of eight months if convenient), and this should be repeated seven years later or sometime during the first few years of attendance in school, and always before one reaches the age of 18 to 20. If there may exist a particular danger of or an anticipated exposure to smallpox and especially to the severer forms, all persons should be vaccinated at least every five years as is done in Japan. Vaccination should be repeated in the event of an outbreak of the disease in the community.

Successful vaccination and revaccination (even only once) performed

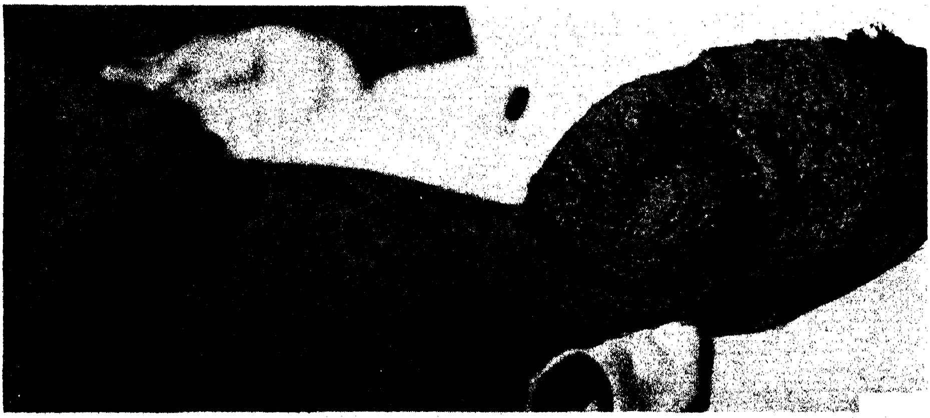
systematically confer complete protection to a community against all forms of smallpox. Even though individual protection may vary, the communal protection against this scourge is practically absolute. A person successfully vaccinated and revaccinated who later is exposed to a severe form of smallpox and contracts the disease has the latter in a much less severe form than unvaccinated persons. The result is that vaccination eliminates completely the milder forms of this disease and not only reduces the incidence rate but lowers markedly the death rate of the severe forms of smallpox.

● The fear of vaccination and the alleged dangers due to this procedure have been greatly magnified and have no basis in fact if simple precautionary measures of cleanliness are observed. Even a pin prick or a knife cut may result in harmful after-effects. We must of course remember that a slight but nevertheless an open wound is produced during vaccination. If a clean or aseptic technique is not used, or if the wound is neglected and dirt gets in, or if unclean dressings are applied, complications and even tetanus as in the case of any other wound may arise. Vaccination wounds in infants are rarely infected and in children they are more likely to become contaminated if the latter are running about or the wounds are freely exposed as during the summer months. Cleanliness is absolutely essential and this will result in the complete elimination of possible septic infections and tetanus.

Autovaccination is the result of the scratching of the virus with the fingers and transferring it to the mucous membranes of the nose, mouth or any part of the skin. Vesicles at these distant places may thus appear. Introduced into the eye, blindness may result.

With the present technique of vaccination where dressings are avoided and the insertion of the vaccine virus is restricted to a very small and superficial area, thus insuring the rapid drying of the vesicle and the primary vaccination is performed in most instances in early infancy, it is exceedingly rare to hear of tetanus or demyelination encephalomyelitis causing complications during vaccination, neither due to the vaccine virus itself.

The possible dangers connected with vaccination where the proper technique is employed are infinitesimal as compared with the benefits derived. If vaccination was made compulsory and was rigidly followed, smallpox would be unknown. It exists in this world, due to the unvaccinated who are a detriment not only to themselves but to everyone in the community.



A virulent case of smallpox in a patient who had never been vaccinated against smallpox.

XXVI

VACCINUM RABIES

RABIES VACCINE

Vac. Rabies, Antirabic Vaccine, Antirabic Virus, Pasteur Treatment, Pasteur Prophylactic

"RABIES Vaccine is a sterile suspension of the attenuated, diluted, dried or dead, fixed virus of rabies. The virus is contained in the tissue of the central nervous system of an animal suffering from, or dead of, fixed virus rabies infection.

Description and Physical Properties

● A more or less turbid, white or whitish liquid, nearly odorless or having an odor due to the presence of a preservative. Rabies Vaccine must come from animals that are healthy excepting for rabies infection. It must not contain an excessive proportion of preservative (not more than 0.5 per cent of phenol or 0.4 per cent of cresol if either of these is used).

Rabies Vaccine must be prepared in an establishment licensed for the purpose by the Secretary of the Treasury of the United States.

The outside label must bear the name *Rabies Vaccine,* the manufacturers' lot number on the Vaccine, the name, address, and license number of the manufacturer, and the date beyond which the Vaccine may not be expected to retain the potency prescribed by governmental authority.

Storage

● Preserve Rabies Vaccine at a temperature between 2° and 10°C., preferably at the lower limit. It must be dispensed in the unopened glass container in which it was placed by the manufacturer.

Average Dose

● The contents of one container, to be repeated at proper intervals."

Rabies or hydrophobia is an acute infectious disease of mammals, most prevalent among carnivora, and caused by a filtrable virus infecting chiefly nervous tissue (neurotropic). The disease is transmitted to susceptible animals by the saliva of an infected animal coming in contact with a broken surface as is possible when the skin is abraded by bites or scratches. Dogs, due to their large numbers and comparative freedom are the ani-

mals most frequently infected and the disease is in turn communicated to man most frequently by the bites of such rabid dogs. In 1903, Negri (*Zeitschr. f. Hyg. u. Infectionskrankh.*, 1903, XLIII, 507; XLIV, 519) described certain characteristic intracellular bodies (peculiar cell inclusions, named *"Negri Bodies")* within the gray nervous tissue of rabid animals. Though the nature of these structures is still under discussion, investigations have fully established the diagnostic value of Negri bodies as specific for rabies. In man, the incubation period of this disease varies from about 15 days to two or even three months and the certainty and rapidity with which rabies may develop are influenced by such factors as (Park & Williams, *Path. Microorg.* 1933, p. 689): (a) The location of the injury and its proximity to the structures of the central nervous system; (b) the depth and extent of the wound; (c) the quantity and virulence of the rabic virus introduced (and whether the bite was through clothing); and (d) the susceptibility of the animal bitten. As in tetanus when the clinical symptoms of rabies appear, the disease process is already well established; and it is invariably fatal to the infected human being or animal, for our body cells do not appear able to produce protective or immune bodies in sufficient quantity or quickly enough to combat the virus. In cases of bites by an animal suspected of being infected with rabies, the animal should be quarantined to determine whether or not rabies is present. The wound should receive surgical attention immediately, including cauterization with fuming nitric acid. If the symptoms in the quarantined animal reveal clinical rabies or even suspicious findings, the Pasteur Treatment should be started as soon as possible without waiting for the laboratory report. This so-called Pasteur Treatment (*l'Etude experimentale de la Rage,* Paris, 1909) is now prophylactically applied to patients infected with or suspected of infection with rabies and its principle and technical details have been altered very little since it was first introduced by Pasteur in 1886. The method is an active immunization and is based upon the principle of stimulating the production of rabic antibodies before the disease sets in, by injecting attenuated virus and administering the latter immediately after being bitten and during the long period of incubation in doses of progressively increasing virulence.

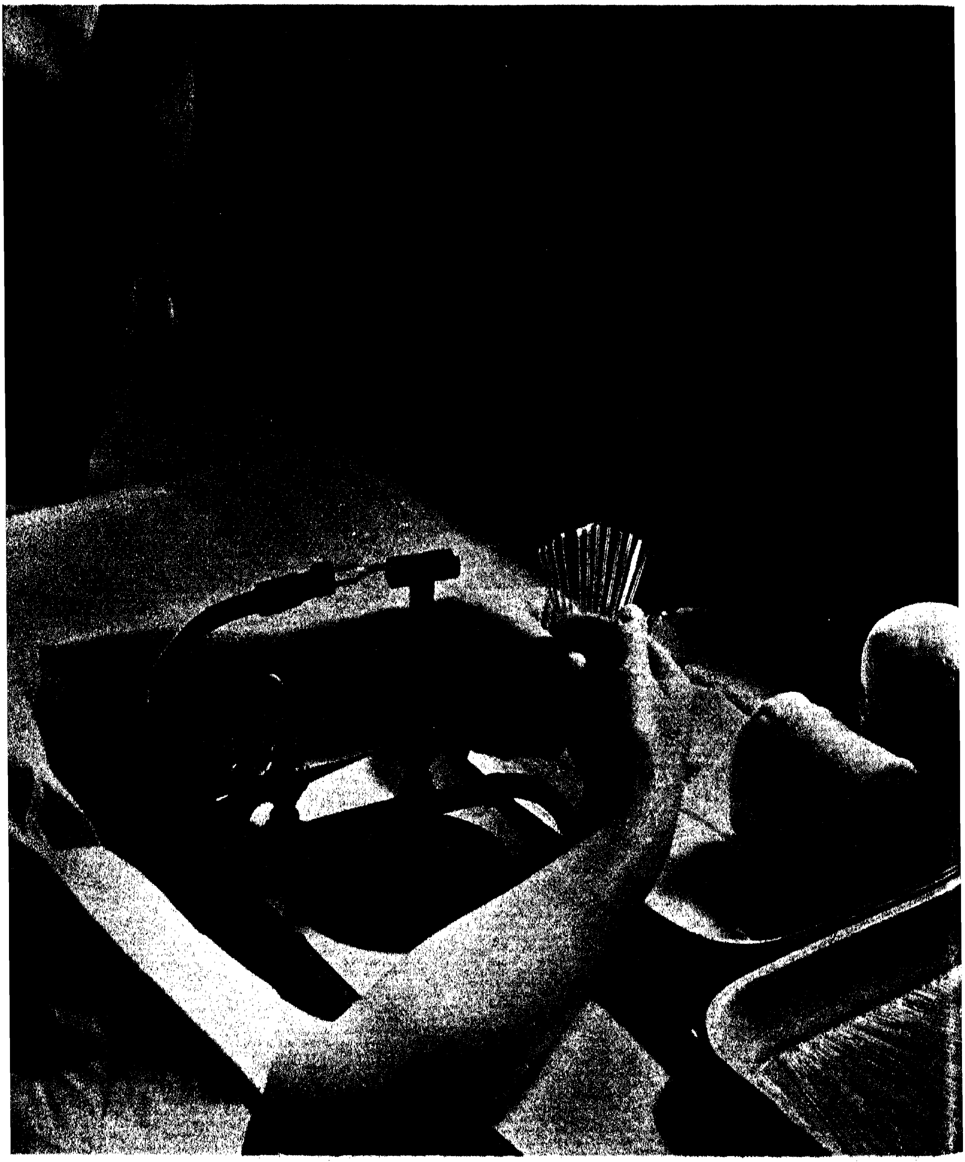
PREPARATION OF RABIES VACCINE

● In preparing Rabies Vaccine or Antirabic Virus, the preliminary step is to obtain *Virus Fixe.* The latter is obtainable from established laboratories. The Paris and Kassauli strains are two well known strains of rabies fixed virus. It is possible to prepare the latter by passing street virus from the medulla (as the virus obtained from natural rabies is known) of a rabic dog through a series of young rabbits (weighing from 700 to 1000 Gm.). The passage in this manner through 25 to 40 rabbits will reduce the incubation period of the virus to seven or eight days, which incubation period then remains fixed (thus the name, *virus fixe).* The spinal cords of rabbits dead due to intracranial injections of emulsified virus fixe are used as the starting point for preparing *Vaccinum Antirabicum.* Various methods are employed for treating these cords (attenuating the virus) either by (drying) suspending them in jars containing caustic potash, (Pasteur's method *(Ann. d. l'Inst. Past.,* 1887, 1888)), or dilution (Hogyes method), emulsification with phenol (Fermi *(Suppl. to Ana. d. l'Igiene,* 1916-26) or Semple *(Brit. Med. J.* 1919, II, 333, 371) method), treating with CO_2 snow and drying in vacuo at very low tempera-



Filling biological containers under aseptic conditions.

Flame-sealing smallpox vaccine capillary tube-points under aseptic conditions.



tures (Harris method *(J. Infect. Dis.* 1921, XXIX, 261), treatment with glycerin (Philips method *(J. Immunol.,* 1922, VII, 409), or formalin, chloroform, or ether (Cunningham's method *(Ind. J. Med. Res.,* 1928, XVI, 245, 253, 259) or by dialyzing (Cumming's method). For further consideration, see *Ind. Med. Res.* Mem. No. 26, 1933. For marketable preparations, pieces of these treated cords are emulsified with sterile saline solution to which phenol, glycerin or other preservative in suitable concentration is added. The concentration of brain and cord tissue varies with the different products. Sterility and safety tests as employed on all other marketable biological products are carried out. The finished product should be kept at refrigerator temperatures (2°C. to 10°C), the expiration date being: "Twenty-one days after emulsification in the case of an emulsion of living virus containing less than 50 percent glycerin; 1 month after emulsification in the case of an emulsion of living virus containing at least 50 percent glycerin; 6 months after date of issue in the case of frozen, desiccated living virus in vacuo, or from date of emulsification thereof; 6 months after date of emulsification in the case of dead virus; the first 7 doses of any form of treatment being considered, for the purpose of dating, as dead virus. Virus known to be living may be kept in cold storage before final preparations as follows: 6 months below 0°C., or 3 months below 5°C., or 6 weeks below 10°C., or 3 weeks below 15°C."

RABIES VACCINE (Semple)

● The use of sterile suspensions of killed brain and spinal cord tissues of rabbits moribund from the injection of Virus Fixe and which confer a high degree of immunity against rabies have come into greater favor within recent years *("A System of Bact.",* 1930, London, H. M. Stationery Office, VII, 198). This is due to the fact that it is unnecessary to employ graduated doses and the possibility does not exist that rabies might be produced as is the case when live rabies virus is employed. Semple's method of preparing such a vaccine is most frequently employed. In this technique an 8 percent emulsified dilution of rabies virus from the brains or brains and cords of rabbits is made in normal saline and it is killed by adding 1 percent phenol and keeping the mixture at 37°C. for twenty-four hours. The emulsion is diluted with saline to yield a 4 percent rabies virus in 0.5 percent phenol. Sterility and toxicity tests are conducted and the suspension is placed in 2 cc. sterile vials. Modifications of the Semple method are also being used. The following technique of preparation is employed by the Laboratories of the New York City Dept. of Health:

"Healthy rabbits are inoculated intracranially with fixed rabies virus as for the preparation of Pasteur vaccine. When the animals are completely paralyzed, they are bled out through the jugular vein, the brains are removed with aseptic precautions and placed in tared Petri dishes. The net weight of the brains is ascertained and an 8% emulsion prepared in 0.8% NaCl solution containing 1% phenol. This emulsion is made by first grinding the brains in a mortar to a uniform paste. While stirring, phenolated saline is added gradually until about two-thirds of the total amount has been used. This mixture is then passed through a Monel metal wire gauze having 100 meshes to the inch. It is an advantage to have the wire gauze held in a frame which fits over the top of the mortar. The remaining saline is used to wash the wire gauze and to dilute the emulsion to the final volume. The emulsion is then passed through a Monel metal wire gauze having 200 meshes

to the inch; it is considered of vital importance that the particles of brain tissue shall be very small in order that the action of the phenol may be uniform and complete. The emulsion, in a bottle, is placed in water at 37°—38°C. for ½ hour (in order to bring it quickly to temperature) and then transferred to the incubator at 37°—38°C. for 24 hours. The exposure of the virus to the phenol at this temperature renders it avirulent for rabbits. An equal volume of sterile saline is added after incubation, giving a 4% emulsion of brain matter containing 0.5% phenol. The dilution is accomplished by forcing saline from a graduated bottle to the stock bottle through sterile rubber tubing by means of air pressure. The air for this purpose is washed through 5% phenol solution and passed through a cotton wool air filter tube.

The diluted vaccine is tested for infectivity by inoculating two rabbits intracranially with 0.5 cc. of the vaccine. The rabbits must survive for 14 days. Sterility tests are made by inoculating fermentation tubes. Transfer is made to fresh fermentation tubes after 7 days incubation. Smears are also made.

The vaccine is filled into vials, 2.5 cc. each, which are closed with no-air stoppers. These final containers are again tested for sterility by the same method as used for bulk tests. All apparatus and containers are sterilized by dry air or autoclave. Grinding, diluting and filling are carried out under a hood. For filling the vials, a special apparatus delivering a uniform quantity is used. The stock bottle is connected to the special apparatus with sterile tubing. The vaccine, after dilution, is stored in the dark at ice box temperature. Vaccine is distributed not later than three months from the date of preparation.

For distribution, 14 vials of vaccine together with a direction sheet, history card and injection record card are packed in a box. The dose is 14 injections on successive days, 2 cc. each."

USES OF (HUMAN) RABIES VACCINE

● Antirabic Vaccine is essentially a prophylactic agent. The efficiency of its use in the so-called Pasteur Treatment (creating an active immunity) in rabies is no longer problematical. When the clinical symptoms of rabies have appeared, the use of this agent as well as that of antirabic serums which have been prepared by immunizing animals and advocated by some in passive immunization are unavailing. There are many methods of immunization in use in different countries so that the specific vaccine and the scheme of treatment are subject to variations according to the individual customs of different workers. It is difficult to say which method is the best *(Am. J. Pub. Health,* 1931, XXIX, 59). Perhaps in the near future, carefully compiled statistics will reveal the technique which yields most satisfactory uniform end-results. The timing of injections are to be from the day when the bite was made. In the treatment of cases of average severity, at least 14 daily injections are given or a total of 14 injections are administered within 14 days after bitten. In severe cases, two daily injections for the first week and one daily injection for the next seven days making a total of 21 injections within 14 days after the infliction of the wound are generally advocated. Some workers use the intensive treatments in all cases *(Ann. Inst. Past.,* 1934, *52:*352). The interval between the daily doses may be arranged and guided by the convenience of both patient and attending diagnostician. It is imperative that rabies vaccine is used in sufficient dosage early in

the course of treatment so that ample time be available for the development or production of maximum protective response. Injections are given subcutaneously into the tissues of the anterior abdominal wall. Avoid the inguinal region and do not penetrate muscular layers. There are no contraindications to the use of this treatment. Children receive the same dose as adults. Exposure to cold, including cold baths, should be avoided during the course of treatment.

There is little data available as to the duration of the immunity after a complete course of rabies vaccine injections but this probably does not last more than one or the maximum two years. An interesting analysis on the results of anti-rabies treatment from all Pasteur Institutes is presented by McKendrick (*Bull. Health Org., League of Nations, 6,* 17 (1937)). In a survey of rabies in Indiana from June 1936 to 1938, the conclusion is reached that there is no reason for modifying the criteria in recommending the "Pasteur Treatment" to persons bitten by or exposed to rabid animals, (*Indiana M. J., 32,* 366 (1939)). On the other hand there are some who feel that the statistics are inadequate to prove the actual value of Rabies Vaccine, and go as far as to state that the Pasteur treatment is not a success.

REACTIONS PRODUCED

● In the use of phenol-killed virus, reactions have been reduced to a minimum and only slight local discomfort is apparent. The appearance of paralytic symptoms following the use of antirabic virus in man has been noticed since the days of Pasteur (*U.S.P.H. Reports,* 1930, XLV, 1888). It seems that when present in the instances noted, regardless of the method of immunizing treatment, these symptoms appear only in adults, from the sixth to the twentieth day after the initial injection, vary in their degree of severity and are known to occur regardless of the type of modified virus used (*J. Hyg.* 1932, XXXII, 33). The etiology of the condition is not definitely known though it has been shown that these paralytic symptoms are characterized by the absence of Negri bodies (*J. Med.,* 1934, xv, 68) in the nervous system.

TREATMENT FOR THOSE BITTEN

Prompt cauterization of the wound should not be neglected. The earlier it is done, the more effective it is. *Fuming nitric acid* is helpful if used within 48 hours after the bite. Apply on the point of a glass-rod or drop by drop from a capillary pipette, so that the amount may be carefully controlled. Contact with bony, cartilaginous or bloodless parts should be avoided if possible. To these parts apply pure carbolic acid and the fuming nitric acid to the other tissues adjacent. Such tissues heal well after the use of nitric acid. The use of pure carbolic acid, iodine, silver nitrate, etc., on wounds made by rabid animals have but little value as compared with fuming nitric acid. Do not suture wounds made by rabid animals.

The Pasteur Treatment is to be given:

To persons bitten by animals which have been proven rabid either by clinical symptoms, or by microscopic examination of the brain.

To persons whose hands or face have been contaminated with saliva of a rabid animal without being bitten. This is because of the possible presence of cracks, hang-nails or other small open wounds.

To persons bitten by stray dogs which cannot be located. The treatment is given as a precautionary measure.

To persons bitten, pending the laboratory diagnosis on the brain of the

biting animal, provided that the symptoms or actions of the animal were suspicious.

A dog which shows *clinical symptoms* suspicious of rabies should be killed at once and the brain sent to a laboratory for diagnosis. If an *apparently normal* dog bites anyone, it should be kept under observation for ten days to three weeks. The saliva of the dog may be infective on an average of three or four days before the animal shows clinical symptoms. The longest time that a dog with symptoms of rabies lives is usually five or six days. This period, added to the three or four days above makes a period of about nine or ten days, at least, during which the animal should be under close observation and confinement. Since exceptionally, a prolonged period between infectiousness of saliva and the development of clinical symptoms may occur, it is safer to hold the animal for three weeks. The microscopic evidence of rabies (the Negri bodies) appears but little earlier than the clinical symptoms. When this evidence in the brain is lacking, a part of the brain must be injected into test animals. This test requires from two to four weeks for completion. It is best, therefore, not to kill an animal before definite clinical symptoms have developed.

CANINE RABIES VACCINE

● The manufacture and distribution of this preparation in interstate commerce as in the case of all veterinary biologicals is under the supervision of the U. S. Bureau of Animal Industry. Though veterinary biological products are not considered in this volume, it is deemed advisable to briefly consider this preparation inasmuch as methods for preventing rabies in dogs will not only save these animals but will aid considerably in reducing the incidence of hydrophobia in man. Kelser, (*Science News Letter, 35,* 393 (1939)), recommends the prophylactic immunization of dogs only as an adjunct in the restraining of dogs in an infected community and in the quarantine of imported dogs. He is of the opinion that the danger of rabies is overemphasized and that the administration of rabies vaccine just to "play safe" is not always advisable. He suggests that dogs suspected of having rabies should be quarantined for observation rather than being shot. Those bitten about the head and face should receive the Pasteur treatment at once. At the end of the ten-day observation period, if the dog proves well, the treatments can be discontinued.

Rabies Vaccine Canine Prophylactic is a phenol killed brain and spinal cord tissue suspension, the tissue being obtained from animals previously inoculated with fixed virus. The tissue content is generally twenty percent. A chloroform-killed 33 1/3 percent tissue content preparation has been used by some.

For immunizing healthy dogs and other domestic animals against infection, a single 5 cc. dose or 10 cc. for very large dogs constitutes the complete treatment. Reimmunization is to be practiced once a year. When used for animals bitten by an infected dog, at least five daily injections are to be given and the bitten animal is to be kept isolated for at least six weeks. Injections are given subcutaneously under the loose skin over the shoulder or back and the dosage is preferably distributed at two or more different sites. It appears that by distributing the dosage administered on the same day, better absorption is apt to occur with the resultant likelihood of proper immunization. Denison and Hunter (*Am. Pub. Health Asso.,* Southern Branch, *Trans. 4th Ann. Meet., 35* (1935)) present their experience in the immunization of 78% of the dog population in a Southern city.

RABIES CULTURE VIRUS

● Webster and Clow (*Science, 84,* 488 (1936)) discuss the propagation of rabies virus in tissue culture and the successful use of culture virus as an antirabic vaccine for the immunization of dogs. They point out that brain tissue, not only a superfluous but a potentially dangerous vehicle, has remained inseparable from the attenuated virus. The disadvantages accompanying the use of animal tissue as a source of virus are largely overcome by the use of culture virus. Experimentation is being continued to prove the effectiveness of this type preparation for use in man and animals.

Webster (*New Eng. J. of Med., 217,* 687 (1937)) discusses further the tissue-culture rabies virus vaccine. He also states that "a conservative point of view should still be maintained in regard to the treatment of human beings with vaccine following exposure." The same author (*Jr. Exp. Med. 70,* 87 (1939)) presents a mouse test for measuring the immunizing potency of antirabies vaccines.

XXVII

YELLOW FEVER

YELLOW fever is an acute disease with as its outstanding clinical features, fever, jaundice, albuminuria and hemorrhages, especially from the wall of the stomach. This malady is caused by a virus which is present in the blood stream. The belief was held for years that the virus of yellow fever was to be found only in man and in the *Aedes aegypti* mosquito neither of which could maintain the virus over long periods of time without the other. Aedes develops only in places where high temperatures prevail and this accounts for the fact that yellow fever is found mainly in tropical and subtropical countries, though transport by aeroplane both of human beings in the incubation period of the disease and of infective mosquitoes involves the risk of disseminating this affection to countries not yet affected. The preventive measures naturally centered around the extermination of the transmitting species of mosquito (*Aedes aegypti* formerly known as *Stegomyia fasciata* or *Stegomyia calopus)*.

Though remarkable progress has been made in the eradication of yellow fever in the Americas, in Africa and in Asia, recent investigations have revealed that there were two unknown and distinct epidemiological factors which were not previously recognized: (1) Rural yellow fever transmitted by *Aedes aegypti* and (2) Jungle yellow fever occurring in the absence of *Aedes aegypti.* Viscerotomy or specifically the routine postmortem removal of liver tissue for microscopical observation from the bodies of all persons dying less than 11 days after onset of any febrile illness irrespective of the clinical diagnosis, revealed the existence of the "silent" endemic areas especially in Brazil (*Trans. Royl. Soc. Trop. Med. & Hyg., 32,* 297, (1938)). The extension of the anti-aegypti measures in the rural areas resulted in the disappearance of yellow fever from these regions. As a result of viscerotomies and the introduction of the mouse-protection test for determining immunity (*J. Exp. Med., 54,* 533, (1931)), followed by field surveys (*Am. J. Trop. Med., 17,* 457, (1937)), there was presented evidence that as for other areas in South America, yellow fever is a jungle infection of which *Aedes aegypti* is not the vector and for the maintenance of which man is not necessarily an important source of virus. This term

"jungle yellow fever" is only of epidemiological significance. Clinically, immunologically and otherwise it has so far been impossible to differentiate it from the classical aegypti-transmitted yellow fever. All factors responsible for maintaining the jungle infection are not definitely known. Whether other species of mosquitoes are vectors or the possibility that certain monkeys are carriers are being considered. At present it is impossible to fit all facts into a simple mosquito-monkey-mosquito-man cycle of infection.

IMMUNIZATION AGAINST YELLOW FEVER

● The use of convalescent human (and even baboon) serum for passive immunization has been used as a prophylactic for those exposed to the risk of infection.

Findlay *(Bull. de l'Office Internat. d'Hyg. Publique,* 1933, XXV, 1009) employing the technique of Sawyer and Associates *(J. Exp. Med.,* 1932, *55:*945) actively immunized individuals against yellow fever.

Workers associated with the Rockefeller Foundation are responsible for the present effective program in the use of agents for active immunization against yellow fever and especially jungle yellow fever. The most efficient product is one designated by them as *Yellow Fever Vaccine Virus (17-D).* Its method of preparation *(Am. J. Trop. Med., 18,* 437 (1938)) details the following which are of interest:

"The 17-D virus came originally from the Asibi strain, a complete history of which has already been reported. After many passages in rhesus monkeys it was established in tissue culture containing mouse embryonic tissue and ten per cent normal monkey serum in Tyrode solution. After cultivation through eighteen passages in this medium, a subculture was initiated in a medium containing whole chick embryo. After 58 passages in whole chick embryo, a subculture was begun in a medium the tissue content of which was chick embryo from which the brain and spinal cord had been removed. The virus has been maintained continuously for over 200 subcultures in this medium containing minimal amounts of nervous tissue.

Preparation of vaccine. As the titer of virus produced by multiplication in tissue culture is low, vaccine for human use is prepared from developing chick embryos inoculated with culture virus. Fresh supernatant fluid from the current culture or virus material preserved in a dried state is used as the inoculum. Only virus cultivated through from 205 to 287 passages has been used in making vaccine. Eggs with living embryos, preferably 7 days old, are selected for inoculation, the number of eggs inoculated varying according to the amount of vaccine to be made. After cleaning the egg shell with 95 per cent alcohol, a small hole is drilled with a sharp dissecting needle directly over the embryo, as shown by candling. Using a tuberculin syringe with a 1-inch 27-gauge needle, the embryo is stabbed by a quick thrust of the needle and 0.06 cc. of undiluted culture material is injected, after which the hole in the shell is sealed with melted paraffin. The inoculated eggs are incubated at 37°C. for three or four days before the embryos are taken out for the preparation of vaccine.

To prepare vaccine, the living embryos are removed from the eggs and pooled. The pooled embryos are weighed and then triturated in a mortar with the aid of an abrasive. A ten per cent suspension of this finely-ground tissue is made, generally in undiluted normal human serum, although serum diluted 50 per cent with distilled water gives satisfactory results. The material is centrifuged for thirty minutes at 3,000 r.p.m., after which the

supernatant fluid is removed and given a preliminary filtration for clarification through a Buchner funnel with a thin film of asbestos fiber. Final filtration is through Seitz E. K. filter discs at ten-pounds' pressure. The vaccine is put up for distribution in ampoules containing 3.0 cc. and in 1.0-cc. tubes. It is then frozen in an alcohol-carbon-dioxide-snow mixture and desiccated in vacuum in desiccators of the Hempel type, which depend on concentrated sulphuric acid for the absorption of water vapor. Each lot of vaccine is tested for bacterial sterility, for virulence, and for quantity of virus. Aerobic and anaerobic tubes of beef infusion broth, containing 0.1 per cent dextrose, and tubes of Hitchen's medium are used. Neurotropism and enhanced viscerotropic properties are tested by intracerebral inoculation of 1 cc. in a rhesus monkey. The quantity of virus is determined, before desiccation and two to three weeks thereafter, by intracerebral inoculation of a number of serial dilutions of the vaccine in a corresponding number of groups of six white mice. From the resulting mortality from encephalitis, the theoretical dilution which should produce a 50 per cent mortality is calculated. The quantity of virus in different lots of vaccine has varied considerably. Some lots had so little virus that they were discarded. The vaccine is stored at a temperature of about 4°C. and is always sent to the field packed in ice in thermos containers.

Technique of vaccination. The vaccination consists of subcutaneous inoculation of rehydrated vaccine, diluted according to the virus content and the dosage of virus desired, at the insertion of the deltoid muscle on either arm. The usual amount inoculated is 0.5 cc., although doses of 1.0 cc. are at times used in the laboratory. The virus is rehydrated immediately before use and the residue after vaccination is inoculated intracerebrally into mice to establish the viability of the vaccine used.

Test for immunity. The intraperitoneal mouse-protection test is used for testing the antibody content of the sera of persons before and after vaccination. This test serves not only to demonstrate the presence or absence of protective antibodies in a serum, but can be used also to determine the quantity of antibody present by the testing of serial dilutions of a serum in several groups of mice. The antibody titer of a serum is expressed as that dilution of the serum which theoretically, under the conditions of the test, should protect one-half of the mice inoculated."

During the year of 1938, more than one million persons, largely in Brazil were immunized with Yellow Fever Virus 17-D. In the 1938 Rockefeller Foundation Review by President Fosdick the following statement is found on page 13:

"The duration of the immunity induced by Virus 17-D can be determined only by future studies. Of twenty-one persons whose blood was tested one year after vaccination, nineteen were found to be still immune. Monkeys vaccinated two and a half years ago are still completely immune. The average duration of post-vaccination immunity must be determined by fundamental investigations before a reasonable vaccination program covering a period of years can be drawn up for exposed populations."

XXVIII

POLIOMYELITIS

ACUTE (anterior) poliomyelitis or infantile paralysis is an acute infectious disease involving the nerve cells of the spinal cord and medulla. The inflammatory process is localized chiefly in the anterior horns of the spinal cord. Inasmuch as lesions occur in other parts of the central nervous system, the term "anterior" is generally dropped. The term "infantile paralysis" is likewise unsuitable as it is known now that many become affected without becoming paralyzed. It is a disease principally of childhood and occurs in epidemic and endemic form. For a complete discussion and survey of this malady see "Poliomyelitis," J. Milbank, Williams and Wilkins, Baltimore, 1932. Morse *(Maine Med. J., 28,* 13 (1937)) presents a detailed summary of a panel discussion in which all problems concerned with poliomyelitis are considered and an interesting treatise along the same lines by MacNalty *(Brit. Med. J., 11,* 57 (1936)) is available.

The etiological agent of poliomyelitis is one of the smallest of known viruses (measuring from 12 to 17 *mu* in diameter). The claims of streptococci by Rosenow and "globoid bodies" by Flexner and Noguchi as causative agents have never been substantiated and are regarded as being without etiological significance. The virus does not grow on lifeless media. The only available experimental animal is certain species of monkeys *(Macacus rhesus* monkeys). In spite of the limitations of experimental work, considerable data is available concerning the properties of the virus *(Proc. Soc. Exptl. Biol. Med., 37,* 160, 166 (1937)). There exists more than one antigenic strain of the virus *(J. Exp. Med., 58,* 513 (1933); *65,* 687, (1937)).

PROPHYLAXIS

● In recent years two methods of specific prophylaxis, active and passive have been introduced. In the consideration of the use of convalescent serum, mention was made of the use of intramuscular injections of convalescent poliomyelitis human serum (5 to 15 cc.) as a prophylactic agent. The clinical impression is that convalescent serum is of some prophylactic value (*J. Pediat., 10,* 111 (1937)) though attempts to evaluate it have not been

sufficiently controlled to give worthwhile statistical findings. The effectiveness if any is of short duration as is to be expected of all antisera *(passive immunization)*. A high percentage of the adult population is immune to the disease and normal adult blood and blood serum have been advocated as prophylactic agents (*J. Prev. Med., 56,* 319 (1932)).

Several vaccines have been recommended recently for use in active immunization against this dreaded malady. These preparations are viruses (in the brain and spinal cords of monkeys) which have been killed or attenuated by treatment with formaldehyde (Brodie Vaccine, *J.A.M.A., 105,* 1089 (1935); (*Science, 79,* 594 (1935) or with sodium ricinoleate (Kolmer Vaccine, *J.A.M.A.,* *104,* 456, (1935); (*105,* 1956, (1935); (*Clin. Lab. Rev. 1,* 47, (1935)) or with immune serum. Kramer (*Mississippi Doctor, 16,* 1 (1938)) in an evaluation of different procedures recommended for the prevention of poliomyelitis states that results with human convalescent serum and the Brodie and Kolmer vaccines have been unsatisfactory. He discusses three vaccines developed by himself and others and believes that greater promise lies in their use. Toomey *(Am. J. Dis. Child., 55,* 1261, (1938)) prepared two types of an experimental antiserum obtained from horses, and Rosenow *(Proc. Staff Meetings Mayo Clinic, 13,* 328 (1938)) prepared an experimental poliomyelitis streptococcic serum-vaccine. Further work is necessary before a suitable vaccine or animal antiserum can be generally recommended as an efficient prophylactic agent. Most workers do not advocate the use of any of the vaccines so far developed.

The more recent line of attack is the use of the chemical treatment of the olfactory mucosa in order to block the route by which the virus passes to the nervous system. Intranasal spraying with (1%) zinc sulfate solutions are advocated (*J.A.M.A., 108,* 2184, (1937); (*110,* 2024 (1938); (*Am. J. Surg., 42,* 178 (1938)). Tisdall and collaborators *(Canad. Pub. Health J., 28,* 523, (1937)) conclude that this procedure is not suitable as a general public health measure. Picric acid (1%) in saline solution (*Calif. and West. Med., 45,* 138 (1936)) and a picric acid-alum solution *(Am. J. Pub. Health, 27,* 103 (1937)) have also been used. This method of attack has not been approved by most health workers.

THERAPY

● The value of convalescent or immune horse serum as an agent in the treatment of poliomyelitis is difficult to analyze. Divergent results have been reported by different workers, (*J.A.M.A., 92,* 385 (1929); (*99,* 1050 (1932); (*Am. J. Dis. Child., 48,* 481, (1934)). Though definite proof of the efficacy of such sera has not been established on account of the impossibility of achieving perfect experimental conditions, and though convalescent serum is not regarded generally as an effective therapeutic agent, some workers nevertheless advocate the early use of convalescent serum in massive doses (*Lancet, 235,* 642 (1938)). On the other hand, Pohlen (*Deut. Med. Wochschr., 62,* 1245 (1936)) reports on the opposition in Germany to the use of convalescent serum. A preliminary report on a proposed International standard antipoliomyelitis convalescent serum is presented by Madsen and Jensen *(Quart. Bull. Health Organiz., League of Nations, 708,* (November, 1936)). In reviewing all available evidence, clinical and experimental, it appears that convalescent serum possesses very little if any benefit in the treatment of acute poliomyelitis.

XXIX

RICKETTSIAL DISEASES OF MAN

THE generic name of Rickettsia has been given to certain minute organisms which in size and nonfiltrability resemble small bacteria but in other characteristics resemble viruses. The organisms are named in honor of Ricketts who in 1909 had seen these bodies in the blood of patients with Rocky Mountain spotted fever. The important pathogenic species for man are *R. prowazeki,* causing typhus fever; *R. rickettsi,* causing Rocky Mountain spotted fever; and *R. quintana,* causing trench fever. In these three diseases, though agglutinative as well as so-called protective antibodies have been demonstrated, other immunological conditions differ in a number of important respects as compared with bacterial diseases.

Spencer and Parker (*U. S. Publ. Health Rep'ts., 40,* 2159 (1925)) were the first to demonstrate the possibility of achieving immunity in Rickettsia diseases when they used potent suspensions of the viscera of infected ticks, ground in a mortar and killed with 0.5% phenol in immunization against Rocky Mountain spotted fever. Weigl (*Bull. Internat. d l'Acad. Polonaise d. Sc. e. r. Lettres,* 1930, p. 25) presented a vaccine made from the suspensions of infected louse intestines for immunization against typhus. This vaccine is not obtainable for large-scale prophylaxis. Zinsser and Castaneda (*Proc. Soc. Exp. Biol. and Med., 29,* 840, (1932)) prepared large quantities of murine Rickettsiae in X-rayed rats and with thick formalinized suspensions made available a product which is being used on a large scale in Mexico. Veintemillas (*J. Immunol., 36,* 339 (1939)) presents an interesting study with this vaccine. Guinea pigs and human subjects were successfully vaccinated against Mexican orchitic typhus fever by an emulsion of formalinized Rickettsiae containing five billion organisms per cc. The vaccine was prepared in rats irradiated with X-rays and inoculated with the "L" (orchitic) strain isolated from a typhus patient. When 12 persons with no previous histories of typhus fever were inoculated with this vaccine, none of them developed typhus fever upon subsequent infection with large doses of typhus material from infected guinea pigs. Three doses of vaccine were used in the human subjects. A single dose in the guinea pigs was sufficient for protection against the orchitic strain, while three doses of the orchitic strain were required for protection against a non-orchitic strain.

IMMUNIZATION AGAINST THE RICKETTSIAL DISEASES

● The use of human convalescent serum as a prophylactic against typhus fever has been recommended *(Compt. Red. Acad. d. Sci., 151,* 598, (1910)). Various methods of active immunization were attempted *(Dent. Med. Wochenschr., 45,* 57 (1919); (*Presse Med., 35,* 503 (1927)) in which modified virulent human or guinea-pig blood was used as the material for vaccination. Either the danger of the latter or its ineffectiveness has resulted in workers not advocating at present the use of such material.

Spencer and Parker (*U. S. P. H. Rep.,* 1925, XL, 2159); (*Archives of Path.,* 1933 XV, 398)) have succeeded in preparing a modified or killed virus or vaccine from infected ticks (the transmitting agent) which appears to afford protection against Rocky Mountain fever that lasts for one tick season.

Weigl *(Die Methoden der Aktiven Fleck-Fieber Immunisierung,* 1930, Cracow) prepared a vaccine for human use as a protective against typhus fever from the intestines of infected lice. The method is not of value for large scale preparation. Zinsser and Castaneda *(J. Exp. Med.,* 1933, *57:* 381, 391) utilized a formalinized peritoneal exudate from infected rats as their vaccine preparation for immunization against the Mexican variety of typhus fever. Dyer and Associates (*Pub. Health Reps.,* 1932, XLVII, 1329) prepared a vaccine from infected fleas and Blanc and Gaud *(J. A. M. A.,* 1935, *105*:524) report success with the use of the virus of the murine type of typhus isolated from rats. See also page 225.

PROTOZOAN INFECTIONS

● Immunization with killed animal parasites or living tissue containing parasites or the use of attenuated organisms has not been sufficiently developed to be regarded of practical value. This volume lists only the products available and briefly presents a consideration of them. Otherwise there is nothing further to be mentioned under this heading. For a general review of immunological observations and reactions in protozoan infections see Talliaferro, *"The Immunology of Parasitic Infections",* Century Company, New York, 1929.

XXX

ALLERGENS

DIFFERENT proteins are fundamentally the agents which produce the symptoms commonly observed in hay fever, asthma, urticaria, angioneurotic edema, and other allergic diseases (Thommen: *Asthma and Hay Fever in Theory and Practice,* 1931). Freeman speaks of this group of symptoms as *"toxic-idopathy."* A multitude of terms has confused the whole subject of *"hypersensitiveness."* Ricket coined the word *anaphylaxis* (without protection). Von Pirquet introduced the word *allergy* (altered reactivity). Other terms are: *Atopy, idiosyncrasy, protein sensitization,* and *hyperergy.*

The different kinds of proteins may vary in the different types of amino-acids which they contain or the relative proportion and intramolecular placement of the latter. This means that the protein from one plant will be different from the protein of another, or from the protein of egg, milk, or any of the species of bacteria. It, therefore, becomes necessary for our body cells to produce antibodies, protecting substances or ferments which will specifically attack the particular foreign protein and usually none other but the specific protein is attacked. It accordingly becomes apparent that symptoms of hives, eczema, asthma, hay fever and other allergic diseases may be caused by almost any kind of protein. The symptoms may become manifest after the eating of certain foods, such as eggs, oysters, clams, strawberries, etc. Others develop these symptoms by coming in contact with the epidermal emanations from many of the lower animals, including horses, rabbits, guinea-pigs and a host of other animals. In like manner inhalation of the proteins from several sources may be the direct cause of hay fever and asthma. The proteins of the pollen grain from all the important pollenating plants may produce the symptoms, or it may be any of the following: Proteins from horse hair or horse dander; the dust from feathers present in pillows or other feathers and hair which may be a constituent of some object in the house, as the fur of animals, felt usually made from rabbit hair, and even the protein from dry glue in furniture. Foci of bacteria and mucoid secretions in the upper and lower respiratory tract, etc. may be the cause of the allergy. Then we have symptoms in other

cases produced by the protein from house and other dusts. Powdered orris root, a constituent of almost all face powders and many cosmetics, may be the exciting cause, or it may be rice powder and other drugs with which an individual comes in contact.

ALLERGIC SKIN REACTION

● The skin test for detecting the sensitive protein is very simple. A small amount of the several purified proteins usually in solution, is applied separately to small scarifications made on the skin (avoid drawing blood), about ½ inch apart (in the cutaneous, percutaneous or scratch method) or one or two drops are injected intracutaneously (intradermally). Controls are made. If the protein is in powder form this is placed on the abrasion, followed by the addition of 1 drop of sterile water, saline solution or 0.25 per cent NaOH solution. Sterile wooden applicators or toothpicks, one for each test, are used to rub the reagents into the scratches. If the patient is allergic or sensitive to the particular protein, a small (½ inch) urticarial, raised area appears in a few minutes or within one-half hour at the point of injection. This lasts for a period varying from several minutes to one and even two hours, or in some instances longer and then disappears. Reactions are noted from 10 minutes to one-half hour after the test. No allergy to a particular protein is indicated by the non-appearance of such reaction after the test is made. The reaction in the intradermal method appears sooner, is more sensitive, and this technique seems to be the method of choice.

The ideal area for skin testing is the outside of the upper arm or the anterior aspect of the forearm, though the skin toward the wrist may be somewhat resistant. In women and children or when a large number of tests are made, the extensor aspect of the thigh or the back is used. In the *Dermal* or *Scratch Method* where a large number of tests can be carried out at one time, a safety razor blade, a scalpel or a needle is employed to make the scratches (a few millimeters in length), and preferably in a direction parallel to the axis of the limb at right angles to the lines of cleavage of the skin. In the *Intradermal Method,* the same syringe may be used, for all injections (usually not more than six to eight at one time), provided the syringe is washed between injections preferably through three vessels containing sterile saline solution, care being taken that the washing is carried out in the same sequence each time.

The positive skin reaction is characterized by wheal formation, erythema and itching. Though the relative size of the wheal area, extent of erythema and itching are of significance for evaluating reactions on a given patient, the reactions produced in different patients cannot be compared unless due regard is given to the kind of extract used, mode of preparation, and the sensitivity of the different patients' skins.

OTHER METHODS OF PERFORMANCE OF TEST

● The mucous membranes may be used for the detection of sensitivity. In the *ophthalmic reaction* a solution or extract of the suspected allergen is placed into the conjunctival sac. A reddening of the inner canthus to a bloodshot eye with marked irritation and even sneezing is a positive reaction.

In the *nasal test,* solutions or extracts or dusts are inhaled, sprayed or deposited in the nasal passages. An increase of mucous secretion and violent sneezing will result in a positive reaction.

Blistering and swelling will result in a positive test in the *oral reaction*

when the suspected protein is applied to the lips and mouth. In positive reactions in the *rectal installation* of suspicious substances, there will be apparent a marked itching and the characteristic symptoms usually produced in the disorder complained of will be apparent.

The *Patch Test* is used frequently to determine susceptibility to external irritants, particularly those non-protein in character; a convenient technique is to place a 3 cm. square of lint in the center of a four or five cm. square of adhesive and in the center of the former, there is placed a 1 cm. square of four-ply gauze well covered with the substance to be tested. The patches are applied to the upper posterior part of the chest which was previously cleansed and asepticized. The leg may be used. The patch is allowed to remain for twenty-four hours. Readings are made at twenty-four, forty-eight and seventy-two hours. Positive reactions reveal a well-defined, infiltrated inflammatory area covered with papulo-vesicles, vesicles or bullae, accompanied by itching. The positive reaction may persist for from several days to two weeks.

PROTEINS FOR DIAGNOSTIC USE

THE problem of detecting the specific protein which may be the cause of the particular symptoms in the individual is frequently a long drawn-out and tedious affair. At times such detection is impossible. This may be due to the fact that the purified proteins from one or several of the substances to which the individual is sensitive have not been separated in a relatively pure state to be used for testing allergic persons. In most instances it is necessary to employ a larger number of proteins in cutaneous (skin) or intracutaneous (intradermal) tests. The proteins which may be used are frequently only selected after a painstaking search into the history of the patient and his environment. Proteins in dried powder as extracts or as pastes are used only for the cutaneous or skin tests, and many tests can be performed at one time. Proteins in dilute solutions are generally used mainly for the intradermal or intracutaneous test, but may be used also for the cutaneous test. Dilutions are always employed when the intracutaneous tests are made and not more than six or eight of the latter are performed at one time. This is to avoid general systemic reactions.

Several different methods of preparation are practiced in the manufacture of the proteins in pure form. The technique depends upon the property of the crude material, its general composition, etc. Extractions are conducted with different solvents, either water, saline solution, buffered saline, diluted glycerin and alcoholic solutions or any of these containing varying strengths of usually 0.1 to 0.2 percent alkali. A 1% sodium chloride solution, or a buffered saline solution (sod. chloride, 0.5 Gm.; potassium dihydrogen phosphate, 0.0363 Gm.; disodium phosphate 0.1431 Gm.; phenol 0.4 Gm.; distilled water qs. 100 cc.), or 33% of the latter mixed with 67% of glycerin (containing 0.5% phenol) or a liquid consisting of 5 percent of dextrose containing 0.5% phenol are most frequently employed as the extracting fluids. If the extractions are not pure protein, they are further treated with different reagents which precipitate the protein. The latter are redissolved and standardized. The methods of standardization vary. This may be to determine chemically the amount of nitrogen or protein present, and then the solutions are diluted to any desired nitrogen or protein content. In some instances, the amount of allergen in solution is expressed in terms

of amount or percentage of solute to solvent and in few cases complement-fixation and other tests are conducted as a means of determining the activity of the finished preparation.

Solution of these proteins used intracutaneously are to be sterilized and placed in sterile containers. Sterilization is usually conducted by the method of bacteriological filtration, and a preservative is added if one was not present in the liquid used in the extraction. Marketable products are tested for sterility, etc., and must meet all governmental requirements.

MARKETABLE ALLERGENS

THE various biological houses market or supply on order most of the following proteins (atopens, allergens or antigens) employed for diagnostic purposes:

Animal (or Epidermal) (Dried or in Solution).— Among the numerous marketable epidermal extracts are to be found the following: camel hair, canary feathers, cat hair, cat dander, chamois skin, chicken feathers, cow (cattle) dander, dog dander, dog, deer and goat hairs, duck feathers, goose and turkey feathers, guinea-pig hair, horse dander, hog, horse, mule, and mouse hairs, horse serum, ostrich, parrot and pigeon-feathers, rabbit dander, rabbit hair and sheep wool. Extracts (in powder and in solution) of practically all furs are marketed. For intradermal testing or in desensitization, liquid extracts are used. For cutaneous testing, powder or liquid extracts are employed.

Bacterial Protein.— Wherever possible tests should be conducted with separate autogenous antigens (proteins) prepared from the different bacteria recovered in pure culture from the nose, sputum, or other foci of infection present in the patient to be tested. Stock antigens (proteins) of practically all of the commonly observed bacteria are marketed. The latter are killed, dried, disintegrated by grinding and marketed in powdered form.

Food Extracts.—These include foods of animal and vegetable origin. Practically every common food has been implicated (*Am. J. Dis. Child.*, 1923, XXVI, 451). With fruits and vegetables that can be peeled, separate extractions are frequently made from the pulp and ground peelings. Many of the manufacturing firms supply in convenient form extracts (in powder or in solution) of most foods, all of the commonly used sea foods, meats, eggs, milk and milk products, all cereals, vegetables, fruits, legumes, condiments, beverages, nuts, etc. They are used as aids in diagnosis and to determine the exciting cause in cases of asthma, urticaria and other forms of protein sensitization and as therapeutic agents.

Miscellaneous Protein Extracts.— Many substances which are not included in any of the other groups are listed here.

Mention was made of the sensitivity to the different kinds of dusts. For diagnostic testing the specific dust will have to be collected and the protein extracted. Each case may, therefore, be a specific problem, so that the dust protein extracts on the market may serve only a limited number of cases. In testing allergy or idiosyncrasy to drugs the latter in powdered form or extracts of them as fluid extracts are applied to abrasions on the skin. In the case of the vegetable drugs fluid extracts may be employed or extracts

may be prepared. The following substances occur so frequently as the important factors in the various manifestations of sensitization, that they are supplied by several firms either in dried powdered form or as extracts from the latter: Cottonseed, flaxseed, glue, hay, henna, kapok, orris root, pine (white and yellow), poison ivy, poison oak, rice powder (polish), senna, silk, tobacco, vanilla and yeast.

Pollen Extracts.—The biologist is interested in pollens. Hay fever is now commonly regarded as a condition of hypersensitiveness or allergy, especially to pollens. The latter are also the cause of various forms of asthma. The pollens causing hay fever may be those of flowers, grasses, shrubs, weeds or other plants (*J. Allergy*, 1932, III, 352, 542; 1933, IV, 87). These are roughly divided into three groups: (a) Those causing early spring hay fever, in which the tree pollens in the air are the main causative factors; (b) those causing early summer fever when the grasses are pollenating; (c) those causing hay fever in the late summer or early autumn when the weeds, especially the ragweed, are shedding their pollens. The trees pollinate in March and April. Those producing the lightest and most abundant pollen belong to the oak family. Ash, cottonwood, the walnuts and the poplars also produce pollens which are widely distributed in the atmosphere during the pollinating season. The largest percentage of all hay fever is caused by the *Gramineae* (grasses—or Timothy grass, Johnson grass, orchard grass, Bermuda grass, red-top grass) for the spring type, and the *Ambrosiaceae* (ragweeds as common ragweed, giant ragweed, Western ragweed and rough marsh elder, Burweed marsh elder and cocklebur) for the fall type (*Pub. Health Rep.*, 1924, *39*, 2422). Speaking broadly, the brightly colored plants are of less interest in this study than those without colored flowerings. The former possess heavy sticky pollen which are carried by insects from plant to plant. Such pollen are rarely present in the air in high concentrations and surely not at any great distance from the plant; so that if one is sensitive to the pollens of this group of insect-pollinated plants (which includes such plants as daisy, goldenrod, rose, sunflower, etc.), by avoiding the immediate vicinity of these, symptoms of hay fever will not appear. The plants without colorful flowerings and not attracting insects possess lighter pollen grains, which are wind-borne. They may be carried many miles and it is mainly such pollens which one cannot avoid that cause most cases of hay fever. When it is considered that a single plant in some instances may shed millions of pollens within twenty-four hours it can be readily seen how concentrated the air can become with the various pollens.

Individuals who are hypersensitive to one of the grasses usually react to several of the latter. Sensitization is frequently multiple and it, therefore, becomes necessary to determine the specific pollens causing the allergy. Cutaneous tests for pollen and other plant allergens are used almost entirely. The different manufacturing firms market a very large variety of pollens and grasses, which are made available in convenient form for immediate use. The manufacture of pollens and pollen extracts require more care perhaps than is necessary when preparing the other allergens. The pollens must be collected in their respective pollinating season under the supervision of a trained botanist. Only the pollinating portion of the plant is treated, and by means of mechanical shakers or other devices the pollens are removed. The latter are collected, sifted through several sieves, then dried or extracts are made. For the technique employed in extraction, see page 229. More recently a 5 percent cresolized dextrose solution was advo-

cated as the best extracting and diluting fluid for pollen therapy (*Jour. Allergy*, 1934, v, 485). In England pollen extracts are prepared by repeatedly freezing and thawing the pollen in normal saline solution. This is sterilized by bacteriological filtration, diluted and 0.5% phenol is added. To determine which pollens to employ for diagnostic tests, the history of the symptoms of the patient will help considerably. Frequently the patient will give a clue by mentioning what he thinks brought on the attack. But a knowledge of the botanical flora in a given locality is frequently an absolute necessity before definite findings are possible. The nature, distribution and abundance of a particular local flora and its seasonal distribution must be known if patients are to be given relief.

The list of pollens (pollen atopens) used in determining the exciting agent in hay fever and pollen asthma is very large. Almost one hundred different pollen atopens or pollen antigens are to be found available on the market today and to this there is to be added the several pollen atopens which are obtained in localized areas by those scientists in these respective territories who have found it advisable to prepare them.

In the case of pollen allergens it is advisable to have available the distribution of trees, grasses, etc., and their pollination during the different seasons in the various parts of the country. The New York Botanical Garden has available considerable data on the North American Flora. Other references are Thomen's "*Asthma and Hay Fever in Theory and Practice,*" Publication No. 401 of the Carnegie Institute at Washington, Lobeck's Physiographic Map, and rainfall and humidity maps of the U. S. Weather Bureau.

GROUP ALLERGENS

● In practice it is not possible at all times to skin test patients with the exceedingly large number of different allergens. Group allergens, five to eight allergens being included in each group, are available and these make possible the shortening and simplifying of the testing procedure. These mixtures are grouped together according to their biologic or genetic relationships or because their association has a basis in clinical experience. A positive group test indicates sensitivity to one or more proteins in that group and tests with the individual members are then performed. Though a negative group test may eliminate all members in that group, this should not interfere with trying an individual allergen in that group should it be suspected.

STANDARDIZATION OF PROTEINS

WHEREVER convenient, especially when preparations of protein extracts are marketed, some method of determining the activity of the preparation and recording its strength should be practised if possible (*J. Immunol.*, 1925, *X*, 445). The most convenient method is to determine the nitrogen content chemically by the Kjeldahl method. Each 0.001 mgm. of nitrogen is spoken of as a unit, so that if a solution has present 0.025 mgm. it is said to contain 25 units. Such a method is accurate and lends itself to an easy interpretation of any dosage.

The "protein unit" is also employed: The nitrogen content per cc. determined by the Kjeldahl method is multiplied by 6.25. This result is divided by 0.001 to obtain the number of "protein units." Cooke and Stull (*J. Allergy, 4,* 87 (1933)) speak of a unit as 0.00001 mgm. protein nitrogen. Some manufacturers standardize their pollen allergens and express the dosage merely in terms of nitrogen content.

In the case of the pollen extracts, where dried weights are not given, some manufacturers regard 1 unit of pollen as corresponding to 0.001 mgm. of dried pollen. It may be necessary to learn from each manufacturer the basis upon which the units of his products are determined. The animal, food and pollen extracts for desensitizing treatment are conveniently marketed in bulk and standardized to contain 250, 500 or 1000 units per cubic centimeter of solution. Pollen extracts are marketed in dosage as high as 10,000 units per cc. These strengths can be employed readily for any scheme of dosage as may be desired.

In few instances some workers standardize allergens or antigens by means of the complement-fixation test.

PREVENTION AND TREATMENT OF SENSITIZATION DISEASES

IF successful in finding out the proteins to which the individual is sensitive then any of the following procedures are resorted to before attempts are made to desensitize the individual (Rackemann, 1931, *Clinical Allergy*, N. Y.).

Merely avoiding a particular food, if the latter is the cause of the allergy, will generally clear up the symptoms in a patient attacked by such proteins. Feather sensitization is relieved by the removal of feather pillows, feather mattresses, feathers from trophies, and even the dust from the bedroom. It is a good practice to advise all sweeping to be done under moist conditions. Individuals sensitive to the proteins from horse hair, horse dander or other animal epidermal emanations need but avoid such animals to remain free from symptoms. Such patients are, however, to be carefully guarded if serum therapy is to be employed upon them. Unless special precautions are observed when injecting serum preparations, marked reactions and even death may occur.

Individuals sensitive to the pollen of the brightly colored plants, weeds, etc., are to avoid coming in contact with or in the immediate vicinity of these plants. The latter can be removed from in and around their immediate environments. Living or sojourning at the seashore during the pollinating season, where the winds are from the ocean, will help many hay fever patients; but it is not always possible to follow such instructions. The avoidance of many pollen and dust granules, orris root present in cosmetics, rice powder present in face powders, even animal emanations, etc., is difficult and frequently impossible. The majority of such sufferers must look for other measures in order to obtain relief. The present method of treatment depends upon the principle of desensitization to obtain the desired results.

Some attempts have been made to desensitize by giving the specific proteins by mouth or in enemata. Oral administration of pollen preparations is not advocated (*J. A. M. A.*, *112*, 1159, 1171 (1939); (*J. Allergy*, *10*, 156, (1939)). The best procedure, however, is the hypodermic method. Briefly, this is performed as follows: After the proteins to which the patient is sensitive have been ascertained by the cutaneous or intradermal tests, extracts of the specific proteins or allergens in question are obtained. Injections of varying dilutions are given about once a week subcutaneously. Very weak doses are first given and then the dosage is gradually increased, varying with each case and depending upon reactions which prevail. Clinical symptoms or absence of relief determine the dosage and number of in-

jections. The latter may vary from eight to thirty injections, being usually more in number when the pollens are injected. In the case of those allergic diseases which recur at the same time each year, desensitization is started early enough so that the last dose of the many injections is administered at least two or more weeks before the expected attack, or before the flowering season of the plants to which the patient is sensitive. Perennial treatment is favored by many as giving the maximum degree and the greatest amount of relief in hay fever. (*J. Allergy, 2,* 39 (1932); *3,* 180, 548, (1932)). In this procedure, the patient receives preseasonal treatment until his sensitivity is decreased and he can tolerate a maximum dose. After the season, semi-monthly or monthly injections of from ¼ to ¾ of this maximum dose are given and as the next season approaches, increased doses are given until the maximum is reached.

LEUCOCYTE EXTRACT

Leucocyte Extract is prepared from the leucocytes of the blood of healthy animals. Pleurisy with effusion is produced in the latter by injecting into the pleural cavity a sterile solution of starch-meat extract or a similar preparation. The sterile exudate thus produced is aspirated under aseptic conditions and the leucocytes (obtained by centrifugalization) are extracted with distilled water. After Berkefeld filtration, the filtrate is tested for sterility and marketed in sterile containers. The preparation may at times possess a reddish color due to traces of hemoglobin which is extracted from the few red blood cells that may be present.

Leucocyte Extract is administered subcutaneously or intramuscularly in 10 cc. doses every four to six hours until 40 to 60 cc. are administered every 24 hours and until the condition of the patient necessitates its use. A complete resume of the use of this preparation is given by Leonard (*J. Med. Soc. N. J.* 1919, *XVI;* 354). Leucocytic extracts injected into individuals infected with various organisms exert distinct though not powerful therapeutic effects (*J. Med. Research,* 1910, *XXII:*3). Such extracts are being advocated in infections of undetermined etiology, in conditions where a leucopenia prevails, in erysipelas, in furunculosis and in staphylococcus infections.

It is known that bactericidal substances can be extracted from leucocytes by various methods (*Centrabl. f. Bakteriol.,* 1905, 1:39; 1908, 144). Known as *"endolysins"* and *"endoferments"* or *"endoenzymes"*, they are present to aid in the digestion of the microorganisms. The efficiency of this preparation is probably due to the presence of these bactericidal substances coupled with a non-specific protein action (*J. Immunol,* 1917, 2). Fleming and Allison (*J. Exp. Path.,* 1922, *III,* 252) have indicated that other body cells found not only in the blood but in the secretions and in other tissues produce substances of a bacteriolytic nature.

NON-SPECIFIC PROTEIN THERAPY

THE use of various proteins, and even non-proteins, has been tried in the treatment of diseases in which the agent employed bears no specific relationship to the nature of the infective process. In other words this un-

related agent can be termed a non-specific antigen. The first observations were more or less accidental. Even bacterial vaccine therapy (which see) owes some of its value to non-specific effects (*Brit. Med. J.*, 1926, I, 815).

The non-specific antigens and agents that were and are still used are: Protein and protein-split products of bacterial and non-bacterial origin, sera from normal human beings and animals, antitoxic and anti-bacterial sera, milk and preparations of milk products, tissue extracts, hypertonic and hypotonic saline solutions, triple distilled water, colloidal metals, etc. The most commonly employed non-specific agents worth mentioning briefly summarized include:

Bacterial Vaccines—stock bacterial vaccines of typhoid bacilli, colon bacilli or triple vaccine. Autogenous bacterial vaccines and bacterial extracts are also employed.

Sera—normal and immune horse or other animal serum; or normal and convalescent human blood or serum.

Exudates, Transudates and Secretions—human milk (whole or fat free milk), human blister serum, and pleural fluid; products of tissue excitation as produced by drugs (turpentine), cautery, X-rays, etc.

Foreign Proteins and Protein Split Products—egg albumin, milk and milk products, as lactalbumin and casein, peptone, deutero-albumose, proteoses, nucleoproteins, salts of nucleinic acid, gelatin, etc.

Altered Blood Proteins—products of hemolysis caused by the intravenous injection of water, hypotonic salt solution, dilute hydrochloric acid (1:1500) and altered proteins due to the injection of iodine, manganese butyrate, sulphur and other colloidal metals.

Tissue Extracts and Enzymes—extracts of leucocytes, tumor autolysates, trypsin, etc.

Intramuscular injection of milk and its products is being used with good results in many skin, genito-urinary and other affections. Intravenous injections of many of the other products mentioned have been advocated in pneumonia, other systemic infections, and different diseases, (Peterson, 1928, "*Newer Knowledge in Bacteriology,*" 1086, Chicago).

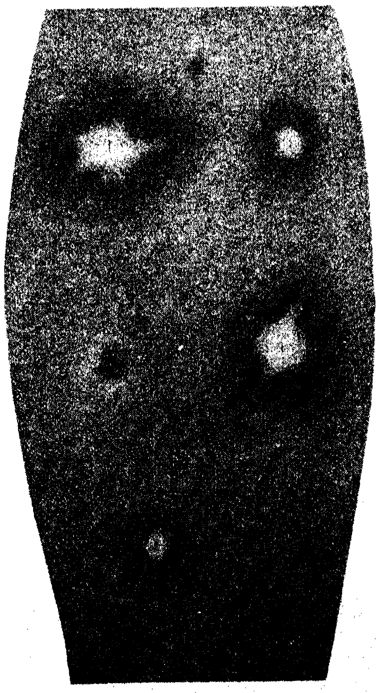
The mechanism underlying the beneficial results of non-specific antigens is not clearly understood. Several theories have been advanced to explain this. No one particular theory is satisfactory, but a combination of some of them affords a satisfactory explanation of the workings of agents in non-specific protein therapy. The response is probably due to the following factors: The blood-making or so-called "hematopoietic" organs, which are probably the producers of many of the antibodies, are markedly stimulated. This results in the throwing forth into the circulation of antibodies already formed, also a marked increase in the production of leucocytes or at least throwing these out in the blood stream in greater numbers, and generally speaking, an increase in our defensive agents are produced. These appear in larger quantities in the circulating blood than are present before the injection of the non-specific agent. It may also be that some of the non-specific ferments, etc., aid in the autolysis of the specific causative agent, or in rendering non-toxic any of the toxic products produced by the latter. So far as known, non-specific agents never produce a specific immunity.

The entire subject of non-specific or "collateral" immunization requires more careful study before this therapy can be recommended for general and wide use (*M.J. and Record,* 1925, *121*:674; 1926, *123*:584). The particular agent, the dose to use, the route of administration to be chosen, and other facts are needed before the relative merits and demerits of

various non-specific agents in the treatment of different diseases can be definitely established. It would seem at present that one is to be guided by the following rule:

For prophylactic immunization, specific agents or antigens are to be employed in an attempt to obtain a specific immunity. For therapeutic immunization (that is in the treatment of disease), specific or a mixture of specific and pseudospecific agents should be tried first. Where these fail or have been found to always fail, the best agent to recommend would be the one which has been found to give the best immunizing response against the specific causative agent which one desires to attack.

Veterinary Biologicals.—There are available as marketable products a considerable number of the various types of biological preparations which are employed as preventives or adjuncts to treatment in many diseases in lower animals (*Am. Vet. Med. Assoc. J., LXXVI,* 6, June 1930, 766). Some of these have been mentioned in the previous pages. Aggressins and bacterial vaccines are employed against blackleg and hemorrhagic septicemia. Bacterial vaccines and therapeutic sera are employed in anthrax, canine distemper, equine influenza, etc. Biological products also have been found of value in bovine abortion, equine abortion, fowl cholera, mastitis, and in other infections in cattle, sheep, fowl, swine or pigs, horses, and dogs. Tetanus Antitoxin (bovine) and Antivenin (veterinary) are also widely used. This volume deals with biological products for human use. For a detailed consideration see the "*Proceedings of the 11th International Veterinary Congress,* London, 1931.



Marked
(+++)
Marked enlargement of wheal, marked erythema. pseudopods marked.

Negative
(—)
No reaction.
Identical with control.

Slight
(+)
Wheal slightly enlarged, slightly irregular in outline, slight erythema.

Moderate
(++)
Wheal moderately enlarged, moderate erythema. Pseudopods distinct.

TYPICAL CUTANEOUS REACTIONS TO THE SCRATCH TEST WITH HAY FEVER POLLENS

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

The author is indebted to the following for the illustrations presented in this volume: Amer. J. Pharm., pp. 58 and 61; Cutter Laboratories, p. 2; Eli Lilly and Company, facing pp. 6, 7, 69, 106, 107, 143, 193, 206, 207 and 214; Wm. S. Merrell Company, facing pp. 7, 68, 107, 142, and 192; Parke Davis and Company, p. 65; and Sharp & Dohme, facing pp. 18, 19, 28, 29, 64, 65, 68, 146, 147, 178, 179, 215 and on pp. 212 and 236.